IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:)	Examiner: Vogel, Nancy T.
)	
Kevin S. BAKER, et al.)	Art Unit: 1636
)	
Application Serial No. 09/944,929)	Confirmation No: 2450
)	
Filed: August 31, 2001)	Attorney's Docket No. 10466/140;
)	P2548P1C21
For: **SECRETED AND**)	
TRANSMEMBRANE)	**Customer No. 23-1925**
POLYPEPTIDES AND NUCLEIC)	
ACIDS ENCODING THE SAME)	

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ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

On October 6, 2004, the Examiner made a final rejection to pending Claims 25-41. A Notice of Appeal was filed on February 2, 2005.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. A request for a 5 month extension of time is filed concurrently herewith.

The following constitutes Appellants' Brief on Appeal.

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1. REAL PARTY IN INTEREST

The real party in interest is Genentech, Inc., San Francisco, California, by an assignment of U.S. Patent Application Serial No. 09/866,028 (the present application is a continuation of this application) recorded on August 29, 2001, at Reel 011913 and Frame 0155.

2. RELATED APPEALS AND INTERFERENCES

The claims pending in the current application are directed to an isolated nucleic acid referred to herein as "PRO361". There exist two related patent applications, (1) U.S. Patent Application Serial No. 10/677,471, filed October 2, 2003 (containing claims directed to the PRO361 polypeptide), and (2) U.S. Patent Application Serial No. 10/735,014, filed December 12, 2003 (containing claims directed to the PRO361 antibody). The 10/677,471 application is also under final rejection from the same Examiner and based upon the same outstanding rejection, an appeal of this final rejection is being pursued independently and concurrently herewith. The 10/735,014 application is currently being prosecuted. A response to a first Office action in that case was submitted on August 3, 2005.

3. STATUS OF CLAIMS

Claims 25-41 are pending in this application.

Claims 1-24, and 42-43 are canceled.

Claims 25-41 stand rejected and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided as Appendix A.

4. STATUS OF AMENDMENTS

There were no amendments to the claims submitted after final rejection. All previous amendments to the claims have been entered.

5. SUMMARY OF THE INVENTION

The invention claimed in the present application is related to an isolated nucleic acid comprising; a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID

NO:83); a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83), lacking its associated signal peptide; to a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO:83); a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO:83), lacking its associated signal peptide; the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or the full-length coding sequence of the cDNA deposited under ATCC accession number 209621 (Claims 27-34).

The invention is further directed to isolated nucleic acids having at least 95% or 99% sequence identity to a nucleic acid sequence comprising the polypeptide shown in Figure 32 (SEQ ID NO:83); to a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83), lacking its associated signal peptide; to a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO:83); to a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO:83), lacking its associated signal peptide; to the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); to the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or to the full-length coding sequence of the cDNA deposited under ATCC accession number 209621, wherein the polypeptide encoded by the nucleic acid is able to inhibit proliferation of stimulated T-lymphocytes. (Claims 25-26).

The invention is further directed to an isolated nucleic acid that hybridizes under high stringency conditions to: a nucleic acid sequence comprising the polypeptide shown in Figure 32 (SEQ ID NO:83); to a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83), lacking its associated signal peptide; to a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO:83); to a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO:83), lacking its associated signal peptide; to the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); to the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or to the full-length coding sequence of the cDNA deposited under ATCC accession number 209621. (Claims 35-37).

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The invention also is directed to a vector comprising one of the above nucleic acid sequences (Claims 38-39) and a host cell comprising the vector (Claims 40-41).

The full-length PRO361 nucleic acid having the nucleic acid sequence of SEQ ID NO:82 is described in the specification, for example, at pages 14-15, Example 34, in Figure 31 and in SEQ ID NO:82. The polypeptide encoded by the PRO361 nucleic acid is described in the specification at, for example, Example 34, in Figure 32 and in SEQ ID NO:83. Page 21, lines 30-33 of the specification, provides the description for Figures 31 and 32. PRO polypeptide and nucleic acid sequence variants having at least about 95% sequence identity with a full length PRO sequence are described in the specification at, for example, pages 23 and 59-62. Hybridization reactions and conditions are described in the specification at, for example, pages 30 and 109-111. Vectors comprising a PRO361 nucleic acid are described in the specification at for example, page 64.

6.   ISSUES BEFORE THE BOARD

I.   Whether Claims 25-41 satisfy the utility requirement of 35 USC §101.

II.   Whether Claims 25-41 satisfy the enablement requirement of 35 USC §112, first paragraph.

7.   GROUPING OF CLAIMS

With respect to Issue I, claims 25-41 stand and fall together.

With respect to Issue II, claims 25-41 stand and fall together.

8.   ARGUMENTS

Summary of the Arguments:

Issue I: Utility

Appellants have previously explained that patentable utility of the PRO361 nucleic acid, which encodes the PRO361 polypeptide, is based upon the ability of the PRO361 polypeptide to inhibit proliferation of stimulated T-lymphocytes, as demonstrated in the Mixed Lymphocyte Reaction (MLR) assay described in Example 34 at page 141 of the present specification.

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Appellants have also submitted with their Response mailed July 12, 2004, the Declaration of Dr. Sherman Fong (the "Fong Declaration"). The Fong Declaration explains that a nucleic acid encoding a polypeptide shown to inhibit T-cell proliferation in the MLR assay would find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases. Accordingly, based on the teachings in the specification and the Fong Declaration, one of ordinary skill in the art would find it credible that the claimed PRO361 nucleic acid, which encodes a polypeptide shown to inhibit proliferation of T-lymphocytes in the MLR assay has utility <u>as encoding an inhibitor of the proliferation of stimulated lymphocytes</u>.

The Examiner rejects this evidence on two bases. First, according to the Examiner "[o]ne of ordinary skill in the art, in possession of these general teachings would recognize that one cannot extrapolate the utility of a compound for suppressing any particular immune response in vivo, from the MLR in vitro assay." (Pages 3-4 of the Office action mailed October 6, 2004). In support of this assertion, the Examiner has cited references by Kahan, "Immunosuppressive therapy." *Curr. Opin. Immunol.,* 1992. 4:553-560; Piccotti *et al.,* "Interleukin-12 (IL-12)-Driven Alloimmune Responses In Vitro and In Vivo." *Transplantation,* 1999. 67(11):1453.1460; and Campo, *et al.,* "Zinc Inhibits the Mixed Lymphocyte Culture." *Biol. Trace Element Res.,* 2001. 79:15-22.

Second, the Examiner argues that even if an activity demonstrated in the *in vitro* MLR assay correlated with an *in vivo* activity, the MLR assay of the present invention still does not demonstrate that the claimed nucleic acids are supported by a specific and substantial, or a well-established utility because according to the Examiner the specification neither discloses that sufficient controls were used nor discloses the specific data of the results of the MLR assay.

Appellants submit that the Examiner applied an improper legal standard when rejecting the claims under 35 U.S.C. § 101 for alleged lack of utility on these two bases. The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration. Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is <u>more likely than not</u> that one of ordinary skill in the art would doubt the truth

of the statement of utility. Only after the Examiner has made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant.

The three references cited by the Examiner do not suffice to make a *prima facie* case that it is more likely than not that no generalized correlation exists between the ability to inhibit proliferation of lymphocytes in the MLR *in vitro* assay and that same ability *in vivo*. Indeed, the teachings of Kahan *et al.* are not particular to the MLR assay nor do Kahan *et al.* examine whether there is a correlation between the *in vitro* activity of a test substance demonstrated in the MLR assay and the activity of that substance *in vivo*. Further, Piccotti *et al.* do not conclude that *in vitro* stimulation of Th1 development *never* correlates with an *in vivo* effect, such as acceleration of graft rejection. Rather, Piccotti *et al.,* conclude that the data collected from the experiments discussed therein indicate that IL-12R is critical for IL-12 driven alloimmune responses both in vitro and in vivo. Moreover, Campo *et al.* also do not teach that there is no correlation between *in vitro* MLR activity and *in vivo* activity. Rather, Campo *et al.,* simply state that their findings *in vitro* still need to be proven *in vivo*. Thus, the combined teachings of Kahan, Piccotti, and Campo do not demonstrate that more likely than not that there is no correlation between the *in vitro* activity of a test substance, as demonstrated in the MLR assay, and the *in vivo* activity of that test substance.

In contrast, Appellants have submitted ample evidence to show that, in general, if a nucleic acid encodes a polypeptide that inhibits proliferation of lymphocytes in the MLR *in vitro* assay, it is more likely than not that the encoded polypeptide would exhibit the same activity *in vivo*. First, the articles by Wolos *et al.,* "Immunomodulation by an inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of *in vitro* and *in vivo* allogeneic responses." *Cell Immunol.* 1993 149(2):402-8; Fung-Leung *et al.,* "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8; Townsend *et al.,* "Combination therapy with a CD4-CDR3 peptide analog and cyclosporine A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplant model." *Clin Immunol. Immunopathol.* 1998 86(1):115-9; Townsend *et al.,* "Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier." *Blood.* 1996

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88(8):3038-47; and Furukawa *et al.,* "Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro." *Clin Immunol. Immunopathol.* 1996 79(1):25-35 (made of record in Appellants' Response mailed July 12, 2004) collectively teach that <u>in general, the ability to inhibit proliferation of lymphocytes in the MLR *in vitro* assay correlates with the same inhibitory activity *in vivo*.</u>

Second, the Declaration of Dr. Sherman Fong, Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc., the assignee of the present application, shows that, <u>in general, a nucleic acid that encodes a polypeptide shown to have the ability to inhibit proliferation of lymphocytes in the MLR *in vitro* assay would be expected to find a practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.</u>

Thus, upon consideration of the totality of the evidence it is clear that, although there may be some examples in the scientific art that do not fit within the central dogma of immunology that there is a correlation between inhibition of proliferation of lymphocytes in the MLR *in vitro* assay and an immunosuppressive effect *in vivo*, these instances are <u>exceptions rather than the rule</u>. <u>In the majority of cases</u>, as exemplified by the articles by Wolos *et al.,* Fung-Leung *et al.,* Townsend *et al.,* and Furukawa *et al.,* and the Fong Declaration, the teachings in the art overwhelmingly show that <u>there is a correlation between the ability of a polypeptide to inhibit proliferation of lymphocytes *in vitro*, as demonstrated by an MLR assay, and the ability of the same polypeptide to achieve an immunosuppressive effect *in vivo*</u>. Therefore, one of skill in the art would reasonably expect in this instance, based on the MLR assay data, that the claimed nucleic acids encode a PRO361 polypeptide that would have utility as an inhibitor of proliferation of lymphocytes. Such a utility is useful in the treatment of diseases where a reduction in the immune response is desired, such as in treatment of autoimmune disorders.

Further, the MLR assay used to identify the utility of the PRO361 polypeptide encoded by the claimed nucleic acid, including the controls and means of analyzing the data gathered from the MLR assay, is both adequately described in the specification and well known in the art. For example, at page 141, the specification describes using CD4-IgG as a control in practicing the MLR assay. One of ordinary skill in the art would appreciate that CD4-IgG is an antibody that

might be used as a negative control by blocking or preventing activation of allogeneic responder cells. Additionally, Appellants disclose that cell culture media can be used as a control. Skilled artisans would appreciate that cell culture media would serve as a control by providing a measure of background levels. Appellants have also incorporated by reference the procedures described in *Current Protocols in Immunology*, unit 3.12. *Current Protocols* teaches that "separate wells with control cultures should be set up that include – for each dose of responder and stimulator cells – replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells." *Current Protocols* also teaches that values obtained from these controls will reflect background proliferation levels. In addition, negative controls, such as wells with either only stimulator cells or only responder cells might be included as a parameter of the MLR assay. Thus, sufficient controls for MLR are both taught by the specification and by references incorporated by reference.

Moreover, Applicants have provided sufficient information regarding how to characterize data gathered in the MLR assay. In particular, the specification makes clear that any decrease below control is a positive result indicating an inhibitory effect, with decreases of less than or equal to 80% being preferred. Further, Dr. Fong attests that it is "his considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, one of skill in the art would expect to find a practical utility when an inhibition of the immune response is desired such as in autoimmune diseases."

Accordingly, when the proper legal standard is applied, one should reach the conclusion that the present application discloses at least one patentable utility for the claimed PRO361 nucleic acid.

Issue II: Enablement

Claims 25-41 stand rejected under 35 U.S.C. §112, first paragraph, allegedly "since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility . . . one skilled in the art clearly would not know how to use the claimed invention." (Page 4 of the Office Action mailed October 6, 2004).

Appellants submit that, as discussed above, a nucleic acid encoding the PRO361 polypeptide has utility in encoding a polypeptide capable of inhibiting proliferation of lymphocytes. Based on such a utility, one of skill in the art would know exactly how to use the claimed nucleic acid, without any undue experimentation.

These arguments are all discussed in further detail below under the appropriate headings.

ISSUE I: Claims 25-28 and 35-40 satisfy the utility requirement of 35 USC §101

The central dispute in this appeal is the utility of the nucleic acids of Claims 25-41. The October 6, 2004, Final Office action maintains rejection of Claims 25-41 under 35 U.S.C. §101 because allegedly "the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility." (Page 2 of the Office action mailed October 6, 2004). This rejection is maintained despite Appellants' assertion of utility at page 141 of the specification, Appellants' arguments demonstrating why that utility is sufficient, numerous art references cited by the Appellants demonstrating a correlation between *in vitro* MLR assay results and *in vivo* activity, and despite an expert declaration attesting that a polypeptide shown to have an inhibitory effect in the MLR assay would be expected to have a practical utility.

Appellants submit, for the reasons set forth below, that the specification discloses at least one credible, substantial and specific asserted utility for the claimed PRO361 nucleic acid.

A.   The Legal Standard for Utility

According to 35 U.S.C. § 101:

Whoever invents or discovers any new and *useful* process, machine, manufacture, or composition of matter, or any new and *useful* improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title. (Emphasis added.)

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In interpreting the utility requirement, in *Brenner v. Manson*[1] the Supreme Court held that the *quid pro quo* contemplated by the U.S. Constitution between the public interest and the interest of the inventors required that a patent applicant disclose a "substantial utility" for his or her invention, *i.e.* a utility "where specific benefit exists in currently available form."[2] The Court concluded that "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion. A patent system must be related to the world of commerce rather than the realm of philosophy."[3]

Later, in *Nelson v. Bowler*[4] the CCPA acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, even though they may not establish a specific therapeutic use. The court held that "since it is crucial to provide researchers with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility."[5]

In *Cross v. Iizuka*[6] the CAFC reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, *i.e.* there is a reasonable correlation there between."[7] The court perceived "No insurmountable difficulty" in finding that, under appropriate circumstances, "in vitro testing, may establish a practical utility."[8]

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[1] *Brenner v. Manson,* 383 U.S. 519, 148 USPQ (BNA) 689 (1966).

[2] *Id.* at 534, 148 USPQ (BNA) at 695.

[3] *Id.* at 536, 148 USPQ (BNA) at 696.

[4] *Nelson v. Bowler,* 626 F.2d 853, 206 USPQ (BNA) 881 (C.C.P.A. 1980).

[5] *Id.* at 856, 206 USPQ (BNA) at 883.

[6] *Cross v. Iizuka,* 753 F.2d 1047, 224 USPQ (BNA) 739 (Fed. Cir. 1985).

[7] *Id.* at 1050, 224 USPQ (BNA) at 747.

[8] *Id.*

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The case law has also clearly established that applicants' statements of utility are usually sufficient, unless such statement of utility is unbelievable on its face.[9] The PTO has the initial burden to prove that applicants' claims of usefulness are not believable on their face.[10] In general, an Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope."[11], [12]

Compliance with 35 U.S.C. §101 is a question of fact.[13] The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration.[14] Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

The well established case law is clearly reflected in the Utility Examination Guidelines ("Utility Guidelines")[15], which acknowledge that an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility." Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that are to be diagnosed.

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[9] *In re Gazave*, 379 F.2d 973, 154 USPQ (BNA) 92 (C.C.P.A. 1967).

[10] *Ibid.*

[11] *In re Langer*, 503 F.2d 1380,1391, 183 USPQ (BNA) 288, 297 (C.C.P.A. 1974).

[12] *See also In re Jolles*, 628 F.2d 1322, 206 USPQ (BNA) 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 USPQ (BNA) 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 USPQ (BNA) 209, 212-13 (C.C.P.A. 1977).

[13] *Raytheon v. Roper*, 724 F.2d 951, 956, 220 USPQ (BNA) 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984).

[14] *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d (BNA) 1443, 1444 (Fed. Cir. 1992).

[15] 66 Fed. Reg. 1092 (2001).

In explaining the "substantial utility" standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility."[16] Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement,[17] gives the following instruction to patent examiners: "If the applicant has asserted that the claimed invention is useful for any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility."

B.   Proper Application of the Legal Standard

Throughout prosecution of the application and claims that are the subject of this appeal, and at page 141 of the specification, Appellants have asserted that the claimed nucleic acid, which encodes the PRO361 polypeptide, is supported by a specific and substantial or a well-established utility based on the ability of the PRO361 polypeptide to inhibit proliferation of T-lymphocytes, as demonstrated in the MLR assay.

MLR is a well-established *in vitro* assay for assessing the ability of a test compound to stimulate or suppress T cell proliferation, and consequently the immune response of an individual. In brief, in an MLR assay, an immune response is produced by mixing T cells from antigenically distinct individuals and allowing them to react with one another in cell culture. The MLR assay is described in standard textbooks, including, for example, *Current Protocols in Immunology*, unit 3.12; edited by Richard M. Cocio *et al.*, National Institutes of Health, published by John Wiley & Sons, Inc., the entire contents of which are incorporated in the present specification by reference at page 141, Example 34.

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[16] MPEP §2107.01.

[17] MPEP §2107 II (B)(1).

MLR has been extensively used and is considered to be the best *in vitro* model available to study graft-versus-host disease and graft rejection. It is well known that the transplantation of tissues or organs between individuals with MHC incompatibilities quickly activates the recipient's immune system, which then attempts to destroy the transplanted tissue or organ. Transplantation across minor histocompatibility loci generally induces a slower response. Physicians analyze the major and minor histocompatibility differences to predict the success of the graft and to adjust the aggressiveness of immunosuppressive therapy. MLR can be monitored qualitatively, for example, by following the incorporation of tritiated thymidine during DNA synthesis, by observing blast formation or by similar methods known in the art.

In addition to being useful for determining histocompatibility, the MLR assay is useful for detecting immunostimulatory or immunoinhibitory activities of molecules like PRO361. Indeed, the MLR assay is widely used and is considered a standard assay for testing drug candidates that are potential immunomodulators. For example, the Gubler *et al.* reference[18], submitted in connection with the Fong Declaration on August 4, 2004, teaches that the MLR assay was key to identifying IL-12 as an immunostimulant of T-lymphocytes. In another example, the ability of tepoxalin, an immunomodulatory compound, to suppress graft-versus-host reaction, was first demonstrated by inhibition of proliferation of stimulated T-lymphocytes in an MLR assay.[19]

The MLR assay is well described in the specification at page 141, Example 34, and in the Fong Declaration. In particular, at page 141, the specification describes the protocol for the MLR assay used in the present invention. For example, at lines 13-26, the specification states:

> More specifically, in one assay variant, peripheral blood mononuclear cells (PBMC) are isolated from mammalian individuals, for example a human volunteer, by leukopheresis (one donor will supply stimulator PBMCs, the other donor will supply responder PBMCs). If desired, the cells are frozen in fetal bovine serum and DMSO after isolation. Frozen cells may be thawed

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[18] *PNAS,* 88:4143-4147 (1991)

[19] *See* Fung-Leung *et al., Transplantation,* 60:362-8 (1995)(submitted with the Amendment and Response mailed August 4, 2004).

overnight in assay media (37° C, 5% CO_2) and then washed and resuspended to $3x10^6$ cells/ml of assay media (RPMI; 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, 1% HEPES, 1% non-essential amino acids, 1% pyruvate). The stimulator PBMCs are prepared by irradiating the cells (about 3000 Rads).

The assay is prepared by plating in triplicate wells a mixture of:
100:1 of test sample diluted to 1% or to 0.1%,
50:1 of irradiated stimulator cells, and
50:1 of responder PBMC cells.

100 microliters of cell culture media or 100 microliter of CD4-IgG is used as the control. The wells are then incubated at 37°C, 5% CO_2 for 4 days. On day 5, each well is pulsed with tritiated thymidine (1.0 mC/well; Amersham). After 6 hours the cells are washed 3 times and then the uptake of the label is evaluated.

In his declaration, Dr. Fong further explains that irradiating the PBMCs results in a population of antigen presenting cells that is mainly comprised of dendritic cells. At paragraph 7 of his declaration, Dr. Fong explains that this is an important step in the MLR assay because:

Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naïve T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells.

Thus, as Dr. Fong explains at paragraph 8:

The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

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Similarly, as used in the present invention, the MLR assay is useful for measuring the ability of a test substance (or "test sample" as it is referred to in the specification at page 141) to "inhibit" the drive of dendritic cells to induce the proliferation of T-cells. Therefore, as used in the present invention, the MLR assay is neither a general predictor of immune response nor a measure of histocompatibility, but rather is a specific assay designed to test the ability of a sample, such as the polypeptide of SEQ ID NO:83 or a polypeptide encoded by the nucleic acid of SEQ ID NO:82, to inhibit the drive of dendritic cells to induce T-cell proliferation.

Thus, to overcome the presumption of truth that an assertion of utility by the Appellants enjoys, the Examiner must make a *prima facie* case establishing that, even in view of the above-discussion, it is more likely than not that one of ordinary skill in the art would doubt the truth of the Appellants' assertion of utility based on the inhibitory activity of PRO361 as demonstrated in the MLR assay. This is a significant burden to overcome because statistical certainty regarding Appellants' assertion of utility is not required to satisfy 35 U.S.C. § 101.[20] Indeed, where, as here, an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed as "wrong" even where there may be some reason to question the assertion.[21] Moreover, a 35 U.S.C. § 101 rejection should only be sustained where the asserted utility violates a scientific principle or is wholly inconsistent with contemporary knowledge in the art.[22] Indeed, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial utility.'"[23] As discussed below, when the legal standard is properly applied, it is clear that the Examiner has not met the burden of establishing that more likely than not one of ordinary skill in the art would question the truth of Appellants' assertion of utility.

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[20] *Nelson v. Bowler*, 626 F.2d at 856-857, 205 USPQ at 883-884.

[21] MPEP § 2107.02.

[22] *In re Gazave*, 379 F.2d 973, 978, 154 U.S.P.Q. 92, 96 (CCPA 1967).

[23] MPEP § 2107.01.

C. A *prima facie* case of lack of utility has not been established

The Examiner rejects Appellants assertion of utility based on results obtained in the MLR assay on two bases. First, according to the Examiner, "there is a poor correlation between *in vitro* results and *in vivo* results using the MLR assay." (Page 4 of the Office action mailed November 2, 2004). In particular, although the Examiner acknowledges that Appellants have cited art references demonstrating a correlation between *in vitro* results and *in vivo* results using the MLR assay, the Examiner maintains that the general teachings in the art show that such a correlation is not generally accepted. The Examiner further argues that "[o]ne of ordinary skill in the art, in possession of these general teachings would recognize that one cannot extrapolate the utility of a compound for suppressing any particular immune response *in vivo* from the MLR *in vitro* assay." (Pages 3-4 of the Office action mailed October 6, 2004).

Second, according to the Examiner, several controls, including screening of each individual lot of a serum source for growth support capabilities and possible HLA antibodies, use of a pool of allogeneic cells to measure maximum response as well as use of an autologous control to ensure low background levels, are necessary for meaningful results in the MLR assay. (Page 4 of the Office action mailed October 6, 2004). The Examiner alleges that these controls are not disclosed in the specification, and therefore, the claimed PRO361 polypeptide is not supported by a specific and substantial or a well-established utility. Further, the Examiner alleges that "[t]he issue of lack of statistical analysis, or the lack of the presence of the data itself, provides further uncertainty in the evaluation of any in vitro effect of the PRO361." (Page 4 of the Office action mailed October 6, 2004).

In support of the first argument, the Examiner relies on articles by Kahan *et al.*, Piccotti *et al.*, and Campo *et al.* Specifically, the Examiner alleges that Kahan *et al.*, teach that "no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy." (Page 4 of the Office action mailed February 12, 2004). The Examiner alleges that Piccotti *et al.*, "demonstrate that IL-12 enhances alloantigen-specific immune function as determined by MLR, but this result *in vitro* does not result in a measurable response *in vivo* (i.e. failure to accelerate allograft rejection." (Page 4 of the Office action mailed February 12, 2004). According to the Examiner, Campo *et al.*, "demonstrate that while zinc suppresses alloreactivity in MLC, it does not decrease

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T-cell proliferation *in vitro* nor produce immunosuppressive effects *in vivo.*" (Page 4 of the Office action mailed February 12, 2004). Based on these three references, the Examiner concludes that <u>in general</u> there is no correlation between *in vitro* MLR assay results and *in vivo* activity.

Even considering these references, the Patent Office has failed to meet its initial burden of proof that Appellants' claims of utility are insufficient. The arguments presented by the Examiner in combination with the Kahan *et al.*, Piccotti *et al.*, and Campo *et al.* articles do not provide sufficient reasons to doubt the statements by Appellants that the claimed PRO361 polypeptide has utility. In particular, the statement by Kahan *et al.* is a generalized statement that is not particular to the MLR assay either in general, or as used in the present invention. Further, Kahan *et al.* do not examine whether results obtained from an *in vitro* MLR assay correlate with results obtained *in vivo.* Rather, Kahan *et al.* generally discuss the efficacy of various immunosuppressive therapies. Moreover, Kahan *et al.* is a 1992 reference. The vast majority of references relied on and cited by Appellants (which are discussed more fully below) are dated after the Kahan reference and teach a direct correlation between *in vitro* results in the MLR assay and *in vivo* activity. Thus, the generalized statement from the Kahan reference does not make it more likely than not that one of ordinary skill in the art would doubt Appellants' assertion of utility, based on activity evidenced in the MLR assay.

Neither does the Piccotti *et al.* reference make it more likely than not that Appellants' assertion of utility would be doubted. Indeed, Piccotti *et al.*, note that previously they "reported that the p40 subunit of IL-12 stimulates alloreactive CD8+ Th1 development <u>both in vitro and in vivo.</u>"[24] Additionally, Piccotti *et al.* do not conclude that *in vitro* stimulation of Th1 development *never* correlates with an *in vivo* effect, such as acceleration of graft rejection. Rather, Piccotti *et al.*, conclude that the data collected from the experiments discussed therein "indicate that IL-12R is critical for IL-12 driven alloimmune responses <u>both in vitro and in vivo,</u> <u>and that IL-12 Rβ2 alone does not transducer IL-12 signaling.</u>"[25] Moreover, the Gubler *et al.*,

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[24] Piccotti *et al.*, "Interleukin-12 (IL-12)-Driven Alloimmune Responses In Vitro and In Vivo." *Transplantation,* 1999. 67(11):1452-60, 1453.

[25] Piccotti *et al.*, "Interleukin-12 (IL-12)-Driven Alloimmune Responses In Vitro and In Vivo." *Transplantation,* 1999. 67(11):1452-60, 1453.

and Peterson *et al.* references submitted by Appellants as attachments to the Fong Declaration on August 4, 2004 show a clear instance where the *in vitro* activity of IL-12 correlates with an *in vivo* immunostimulatory effect. Specifically, at paragraph 9 of his declaration, Dr. Fong declares:

> Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991)(Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)]. They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulate capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190(11), 1669-78 (1999)(Exhibit E)].

Thus, taking this evidence as a whole, as it must be taken, the Piccotti *et al.* reference, either alone or in combination with the Kahan *et al.* reference does not make it more likely than not that one of ordinary skill in the art would doubt the truth of Appellants' assertion of utility.

Furthermore, even the Campo *et al.* reference relied on by the Examiner does not teach that there is no correlation between *in vitro* and *in vivo* activity. Rather, Campo *et al.* merely states that the *in vitro* results observed in the experiments discussed therein had yet to be proven *in vivo*. Moreover, Campo *et al.* recognize that "[t]he mixed lymphocyte culture (MLC) is a well-established and important tool for determination of compatibility between host and donor in

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transplantation medicine, <u>as it serves as an in vitro model for allogenic reaction</u>."[26] Even further, Campo *et al.* do not use the MLR (or MLC) assay to measure T-cell proliferation or inhibition, but rather to measure cytokine release. Therefore, the results of Campo *et al.* are not directly applicable to the present situation. Of note though, Campo *et al.* disclose that the test substance, zinc, did not either inhibit proliferation of T-cells in the MLC or produce immunosuppressive effects *in vivo*. Moreover, Campo *et al.* acknowledge that: "[i]n vivo substances like cyclosporine A or FK506 are broadly applied, as they are <u>capable of prolonging graft survival. In vitro, they show an inhibitory effect on T-cell proliferation in the MLC</u>."[27] Thus, when the parameters of the Campo reference that are relevant to the present invention are examined, Campo *et al.*, even in combination with the teachings of Piccotti *et al.*, and/or Kahan *et al.*, does not make it more likely than not that one of ordinary skill in the art would doubt Appellants assertions of utility.

Moreover, the combined teachings of Kahan *et al.*, Piccotti *et al.*, and Campo *et al.* are not directed towards correlation of *in vitro* and *in vivo* activities. Therefore, Appellants submit that the Examiner's reasoning is based on a misrepresentation of the statements and scientific data presented in the above cited reference and application of an improper, heightened legal standard. In fact, contrary to what the Examiner contends, as discussed more fully below, the art indicates that, if a test substance indicates a particular activity, such as the ability to inhibit proliferation of lymphocytes, in the MLR *in vitro* assay, it is more likely than not that the test substance will also demonstrate that activity *in vivo*.

In support of the second basis for rejecting the claims for alleged lack of utility, the Examiner relies on references by Stites *et al.* (eds.) and Rose *et al.* (eds.).[28] According to *Basic & Clinical Immunology* (Stites *et al.*), "MLC is typically used for determining histocompatibility in an individual and as a test for immunocompetence of T cells in patients with

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[26] Campo *et al.*, "Zinc inhibits the Mixed Lymphocyte Culture." *Biological Trace Element Research,* 2001. 79:15-22,15-16 (emphasis added).

[27] *Id.* at 20 (emphasis added).

[28] *See Basic & Clinical Immunology,* Eighth Edition. Stites *et al.*(eds), Appleton & Lange, Norwal, CT (1994) and *Manual of Clinical Laboratory Immunology,* Sixth Edition. Rose *et al.* (eds), ASM Press, Washington, DC (2002).

immunodeficiency disorders. [And] [W]hen running the MLC assay for determining histocompatibility for transplantation, autologous controls combining self with irradiated self are necessary to normalize the response of each cell to stimulators." (Page 5 of the Office action mailed February 12, 2004). The Examiner further argues that "there is known inherent variability of individual cellular responses from day to day which requires performing the entire familial MLC at one time in the case of determining histocompatibility for transplantation." (Page 5 of the Office action mailed February 12, 2004).

Appellants respectfully submit that the controls discussed by the Examiner in the Office action mailed February 12, 2004, are more important when the MLR assay is used as a measure of histocompatibility for eventual transplant operations, but less important when the MLR assay is used, as here, to test the ability of a substance to stimulate or inhibit the proliferation of T-lymphocytes. Further, sufficient controls for the MLR assay are both known in the art and taught in the specification, as discussed more fully below.

Thus, Appellants respectfully submit that the Examiner has not established a *prima facie* case of lack of utility on either basis and respectfully request that this improper ground of rejection be withdrawn.

D. It is "more likely than not" that the claimed nucleic acids are supported by a specific and substantial or a well-established utility

However, even if a *prima facie* case of lack of utility were made on either (or both) of the bases argued by the Examiner, the evidence submitted by Appellants clearly rebuts such a case. With regard to the Examiner's first basis for alleging lack of utility, Appellants have submitted substantial evidence demonstrating that generally, if a test substance shows the ability to inhibit proliferation of lymphocytes in the MLR *in vitro* assay, it is more likely than not that the test substance will exhibit the same inhibitory activity *in vivo*. With regard to the Examiner's second basis for alleging lack of utility, Appellants also have demonstrated that sufficient controls were both disclosed in the specification and known in the art. Additionally, sufficient data and guidance are provided which allow one of ordinary skill in the art to evaluate the conclusions that the PRO361 polypeptide is encoded by the claimed nucleic acid and inhibits T cell proliferation

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in the MLR assay.

In rebuttal of the Examiner's first argument, Appellants rely on paragraph 9 of the Fong Declaration (made of record in Appellants' Response mailed August 4, 2004), quoted above. As discussed above, paragraph 9 of the Fong Declaration discusses IL-12 as an example of an immunomodulatory compound that was first identified in the MLR assay and that was later confirmed to have an immunomodulatory effect *in vivo.*

Indeed, the Gubler *et al.* reference, submitted on August 4, 2004 as an attachment to the Fong Declaration, describes first identifying IL-12 in the MLR assay. In particular, at column 1, page 4143, Gubler *et al.* teach "we initiated a search for a novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo.*" Thus, the Fong Declaration and the references attached thereto (*e.g.* the Gubler *et al.* reference) clearly demonstrate that one of ordinary skill in the art, in general, would believe it to be more likely than not that a molecule shown to have an immunomodulatory effect *in vitro* also would have such an effect *in vivo.*

In addition to the Fong Declaration and references attached thereto, Appellants also have submitted and rely on a 1993 article by Wolos *et al.,* which reports that MDL 28,842 (a S-adenosyl-L-homocysteine hydrolase inhibitor, (Z)-5'-fluror-4',5'-didehydro-5'-deoxyadenosine) inhibited the generation of cytotoxic T-cells in the *in vitro* MLR assay with an IC50 of less than 0.1 microM. Wolos *et al.* further report that the observed *in vitro* results correlate with *in vivo* activity. In particular, *in vivo,* MDL 28,842 inhibited the increase in popliteal lymph node weight induced by injection of allogeneic spleen cells. MDL 28,842 was also evaluated in an *in vivo* model of graft rejection. It was shown that skin allografts on animals given MDL 28,842 survived for 12.2 days, compared to 8.7 days for control animals.[29]

Similarly, Appellants rely on a 1995 article by Fung-Leung *et al.,* which reports a direct correlation between the observed inhibitory activity of tepoxalin in the MLR assay and inhibitory activity of tepoxalin *in vivo.* In particular, Fung-Leung *et al.* report that tepoxalin suppressed

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[29] Wolos *et al.,* "Immunomodulation by an inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of *in vitro* and *in vivo* allogeneic responses." *Cell Immunol.* 1993 149(2):402-8.

murine T-cell proliferation in the MLR assay and that this *in vitro* inhibitory activity correlated with an *in vivo* suppression of graft-versus-host disease. Specifically, Fung-Leung *et al.* report that oral administration of tepoxalin to mice suppressed local graft-versus-host disease by about 40%.[30]

Appellants further rely on another art reference by Townsend *et al.*, which also demonstrates the correlation between *in vitro* MLR activity and *in vivo* activity. Specifically, Townsend *et al.*, reported in 1998, that a CD4-CDR3 peptide analog exhibited similar results both *in vitro* in MLR assays and *in vivo* in analyses of the effectiveness of the peptide analog at preventing graft-versus-host disease.[31]

Thus, the inhibition of T-cell proliferation by the PRO361 polypeptide encoded by the claimed nucleic acid, as seen in the MLR results described at page 141 of the specification, would be expected to correlate with *in vivo* results. The articles discussed herein collectively teach that in general, *in vitro* activity demonstrated in an MLR assay correlates with an *in vivo* effect. Significantly, as discussed above, the law does not require the existence of a strong or linear correlation between *in vitro* and *in vivo* activity. Nor does the law require that such a correlation "always" be observed.[32] Hence, taken in total, although there may be some examples in the scientific art that do not fit within the central dogma of immunology that there is a correlation between MLR *in vitro* assay results and *in vivo* activity, these instances are exceptions rather than the rule. In the majority of cases, the teachings in the art, as exemplified by Gubler *et al.*, Wolos *et al.*, Fung-Leung *et al.*, Townsend *et al.*, and Furukawa *et al.*, the Fong Declaration, and the references cited therewith, overwhelmingly show that an activity demonstrated in the *in vitro* MLR assay correlates with *in vivo* activity. Thus, one of skill in the art would reasonably

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[30] *See* Fung-Leung *et al.*, "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8.

[31] Townsend *et al.*, "Combination therapy with a CD4-CDR3 peptide analog and cyclosporine A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplant model." *Clin Immunol. Immunopathol.* 1998 86(1):115-9. *See also* Townsend *et al.*, "Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier." *Blood.* 1996 88(8):3038-47; Furukawa *et al.*, "Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro." *Clin Immunol. Immunopathol.* 1996 79(1):25-35.

[32] *See e.g., Nelson v. Bowler,* 626 F.2d at 856-857, 205 USPQ at 883-884.

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expect in this instance, based on the MLR data demonstrating that the PRO361 polypeptide (which is encoded by the claimed nucleic acid) has an inhibitory effect on lymphocyte proliferation *in vitro,* that PRO361 polypeptides encoded by the claimed nucleic acids will have an inhibitory effect on lymphocyte proliferation *in vivo.* Accordingly, Appellants submit that the PRO361 polypeptides and nucleic acids have utility in the treatment of immune response, for example in autoimmune diseases.

In rebuttal of the Examiner's second basis for rejecting the claims for alleged lack of utility, Appellants rely on the description of the MLR assay, and the controls used therein. In particular, at page 141 of the specification, Appellants disclose that CD4-IgG may be used as a control in practicing the MLR assay in connection with the present invention. One of ordinary skill in the art would appreciate that CD4-IgG is an antibody that might be used as a negative control by blocking or preventing activation of allogeneic responder cells. Additionally, Appellants disclose that cell culture media can be used as a control. Skilled artisans would appreciate that cell culture media would serve as a control by providing a measure of background levels.

Even further, Appellants also have incorporated by reference the procedures described in *Current Protocols in Immunology*, unit 3.12.[33] *Current Protocols* teaches that "separate wells with control cultures should be set up that include – for each dose of responder and stimulator cells – replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells." *Current Protocols* also teaches that values obtained from these controls will reflect background proliferation levels. In addition, negative controls, such as wells with either only stimulator cells or only responder cells might be included as a parameter of the MLR assay. Thus, contrary to the Examiner's argument, Appellants have disclosed, by incorporating the disclosures of the *Current Protocols* reference, autologous controls combining self with irradiated self for normalizing the response of each cell to stimulators. Appellants therefore, respectfully submit that sufficient controls for MLR are both known in the art and disclosed by Appellants, such that meaningful results can be achieved using this assay.

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[33] *Current Protocols in Immunology,* Cocio *et al.* (ed), John Wiley & Sons, Inc. (1991) (Units 3.12.1-3.12.13).

In rebuttal of the Examiner's argument that the specification fails to provide any data or evidence of the results of the assay sufficient for one of skill in the art to evaluate the conclusion, Appellants rely on the following statements at page 141 of the specification:

> This example shows that one or more of the polypeptides of the invention are active as inhibitors of the proliferation of stimulated T-lymphocytes. Compounds which inhibit proliferation of lymphocytes are useful therapeutically where suppression of an immune response is beneficial. . . . Any decreases below control is considered to be a positive result for an inhibitory compound, with decreases of less than or equal to 80% being preferred. However, any value less than control indicates an inhibitory effect for the test protein. The following polypeptide tested positive in this assay: PRO361.

According to the Manual of Patent Examining Procedure (the "MPEP"), Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a <u>legitimate basis to doubt the credibility of such a statement</u>.

Further, Dr. Fong attests that it is "his considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, one of skill in the art would expect to find a practical utility when an inhibition of the immune response is desired such as in autoimmune diseases." The case law has clearly established that in considering affidavit evidence, the Examiner must consider all of the evidence of record anew.[34] "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument"[35] Furthermore, the Federal Court of Appeals held in *In re Alton*, "We are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner"[36]. Appellants also respectfully draw the

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[34] *In re Rinehart*, 531 F.2d 1084, 189 USPQ (BNA) 143 (C.C.P.A. 1976) and *In re Piasecki*, 745 F.2d. 1015, 226 USPQ (BNA) 881 (Fed. Cir. 1985).

[35] *In re Alton*, 37 USPQ2d 1578 (Fed. Cir 1966) at 1584 quoting *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992)).

[36] *In re Alton, supra.*

Examiner's attention to the Utility Examination Guidelines[37] which state, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered." The statement in question from an expert in the field (the Fong Declaration) states that "it is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases." Therefore, barring evidence to the contrary regarding the above statement in the Fong Declaration, this rejection is improper under both the case law and the Utility guidelines.

For all the reasons discussed herein, no legitimate basis for doubting the credibility of Appellants' statement of utility exists. Therefore, Appellants respectfully submit that the present specification clearly describes a patentable utility for the claimed invention. Accordingly, Appellants respectfully request reconsideration and reversal of the rejection of Claims 25-41 under 35 U.S.C. §101.

ISSUE II: Claims 25-41 satisfy the enablement requirement of 35 USC §112, first paragraph.

Claims 25-41 stand rejected under 35 U.S.C. §112, first paragraph, allegedly "since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention." (Page 4 of the Office Action mailed October 6, 2004).

In this regard, Appellants refer to the arguments and information presented above in response to the outstanding rejection under 35 U.S.C. § 101, wherein those arguments are incorporated by reference herein. Appellants respectfully submit that as described above, the claimed nucleic acids, which encode a PRO361 polypeptide shown to inhibit proliferation of lymphocytes in an MLR assay, have a utility based on inhibition of proliferation of lymphocytes

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[37] Part IIB, 66 Fed. Reg. 1098 (2001).

in the MLR assay. Indeed, one of skill in the art would know how to use the claimed nucleic acids encoding polypeptides that inhibit the proliferation of stimulated T-lymphocytes, for treatment of the immune response, such as in autoimmune disease, without undue experimentation.

Further, Appellants respectfully submit that consideration of the *Wands* factors, set forth in *In re Wands*[38] and discussed on a factor-by-factor basis below, clearly indicates that the present specification enables one of ordinary skill in the art to make and use the claimed invention without undue experimentation.

1. The Nature of the Invention

The first *Wands* factor analyzed is the nature of the invention. The nature of the invention is discussed above at pages 2-4.

2. The State of the Prior Art

The second *Wands* factor is the state of the prior art. The Examiner contends that the state of the prior art regarding the correlation between the ability of a protein to inhibit lymphocyte proliferation in the MLR assay and the ability of the same protein to have an immunosuppressive effect *in vivo* is unpredictable because MLR is an artificial *in vitro* system and does not indicate for what specific conditions and for which specific diseases the claimed invention would predictably function. (Page 3 of the Office action mailed October 6, 2004).

Appellants respectfully disagree. As discussed above, MLR is a well-established assay and in the present invention this assay is used for assessing the ability of a test compound to stimulate or inhibit T cell proliferation. Further, as discussed above, the MLR assay is described in standard textbooks, including *Current Protocols in Immunology*, and in numerous journal articles. Indeed, the art recognizes that: "Reactivity in the MLC probably reflects the initial immune recognition step of graft rejection *in vivo*."[39] Therefore, it is well-known in the art what

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[38] 858 F.2d 731, 737 (Fed. Cir. 1993).

[39] *Basic & Clinical Immunology*, Eighth Edition. Stites *et al.* (eds), Appleton & Lange, Norwal, CT, p. 246 (1994).

conditions and diseases can be treated based on results obtained with a compound in the MLR assay. For example, many of the references cited by Appellants and discussed above, demonstrate that a compound shown to inhibit proliferation of T-lymphocytes in the MLR assay, achieves an immunosuppressive effect *in vivo*.[40] One of ordinary skill in the art would appreciate that such an effect would be useful, for example, in the treatment of graft-vs-host disease.

3.   *Relative Skill of Those in the Art*

The level of skill in the art is high. For example, at the effective filing date, the skilled artisan in the field of Immunology and Immunotherapeutics would likely be a person with a Ph.D. or M.D. degree, sometimes both, and with extensive experience. Such a person would, in fact, find it routine to carry out *in vivo* analyses using the PRO361 polypeptide encoded by the claimed nucleic acids, based on the guidance provided about the PRO361 nucleic acids and polypeptides in the present application.

4.   *Level of Predictability in the Art*

The art of the claimed subject matter is predictable. According to MPEP 2164.01(b), "the 'predictability or lack thereof' in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention." As discussed above at pages 20-22, numerous compounds shown to have an immunomodulatory effect in the *in vitro* MLR assay, have been shown to have a related immunomodulatory effect *in vivo*.[41]

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[40] *See, e.g.,* Fung-Leung *et al.,* "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8.

[41] *See also* Wolos *et al.,* "Immunomodulation by an inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of *in vitro* and *in vivo* allogeneic responses." *Cell Immunol.* 1993 149(2):402-8; Fung-Leung *et al.,* "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8; Townsend *et al.,* "Combination therapy with a CD4-CDR3 peptide analog and cyclosporine A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplant model." *Clin Immunol. Immunopathol.* 1998 86(1):115-9; Townsend *et al.,* "Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier." *Blood.* 1996 88(8):3038-47; Furukawa *et al.,* "Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro." *Clin Immunol. Immunopathol.* 1996 79(1):25-35.

5.   *Presence of Working Examples*

In re Wands also requires the Examiner to consider the presence or absence of working examples.[42] Appellants describe the working MLR assay example at page 141 of the specification. However, the Examiner argues that this example is not sufficient because there is no correlation taught or well known in the art between the MLR assay and *in vivo* treatment of diseases involving the immune response. (Page 3 of the Office action mailed October 6, 2004).

For the reasons discussed above, Appellants respectfully disagree that the MLR assay example provides insufficient guidance. In particular, as discussed more fully at pages 20-22, numerous *in vitro* MLR assay results that show either inhibition or stimulation of T cell proliferation by a test substance, as does the MLR in the present invention, have been confirmed *in vivo*. Indeed, (also as discussed above), in paragraph 9 of his declaration, Dr. Fong declares:

> For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991)(Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)].

Thus, Applicants respectfully submit by describing the MLR assay at page 141, Applicants have disclosed sufficient working examples.

5.   *Breadth of the Claims*

The claims are not overly broad. Instead all of the claimed nucleic acids share a utility based on encoding a polypeptide that inhibits proliferation of stimulated T-lymphocytes. In particular, the claims are directed to nucleic acids with at least 95% sequence identity to SEQ ID NO: 82, or to a nucleic acid encoding the polypeptide of SEQ ID NO:83, wherein any claimed nucleic acids that share at least 95% sequence identity to SEQ ID NO:82 or to a nucleic acid encoding the polypeptide of SEQ ID NO:83 encode a polypeptide that inhibits proliferation of

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[42] *Wands* 858 F.2d at 737.

stimulated T-lymphocytes.

6. *Amount of Direction or Guidance*

Significantly, the claims do not require that the polynucleotide be used to treat a patient. Given that the MLR assay is a well-known assay, as discussed above, one of skill in the art will know how to use the disclosed PRO361 nucleic acids and polypeptides in connection with this assay. The results of the assay alone provide one of skill in the art with valuable information to use in identifying compounds with important pharmacological properties that may be used to treat various immune response conditions, such as graft-versus-host disease.

7. *Quantity of Experimentation*

The *Wands* factors are analyzed as a tool to determine whether undue experimentation is required to practice a claimed invention. However, the Federal Circuit has stated that:

> [t]he determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art (citations omitted). The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

Given the established utility of a MLR assay as used in the present invention, the level of skill in the art, and the significant disclosure found in the specification, any experimentation that might be required to practice the claimed invention would be routine and not undue.

Accordingly, Appellants respectfully request reconsideration and reversal of the enablement rejection of Claims 25-41 under 35 U.S.C. §112, first paragraph.

9. <u>CONCLUSION</u>

For the reasons given above, Appellants submit that the specification discloses at least one patentable utility for the PRO361 nucleic acid of Claims 25-41, and that one of ordinary skill in the art would understand how to used the claimed nucleic acids, for example based on the

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characteristic that the claimed nucleic acids encode a polypeptide that inhibits the proliferation of stimulated T-lymphocytes. Therefore, claims 25-41 meet the requirements of 35 USC §101 and 35 USC §112, first paragraph.

Accordingly, reversal of all the rejections of claims 25-41 is respectfully requested.

Please charge any additional fees, including fees for additional extension of time, or credit overpayment to Deposit Account No.23-1925 (referencing Attorney's Docket No. 10466/140).

Respectfully submitted,

Date: September 2, 2005

By: _____
C. Noel Kaman (Reg. No. 51,857)

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(312) 321-4200

Claim 25: An isolated nucleic acid having at least 95% nucleic acid sequence identity to:

(a)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83);

(b)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

(c)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83);

(d)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

(e)   the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82);

(f)   the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or

(g)   the full-length-coding sequence of the cDNA deposited under ATCC accession number 209621;

wherein the polypeptide encoded by the nucleic acid is able to inhibit proliferation of stimulated T-lymphocytes.

Claim 26 (currently amended): The isolated nucleic acid of claim 25 having at least 99% nucleic acid sequence identity to:

(a)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83);

(b)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

(c)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83);

(d)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated

signal peptide;

(e)   the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82);

(f)   the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or

(g)   the full-length-coding sequence of the cDNA deposited under ATCC accession number 209621;

wherein the polypeptide encoded by the nucleic acid is able to inhibit proliferation of stimulated T-lymphocytes.

Claim 27: An isolated nucleic acid comprising:

(a)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83);

(b)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

(c)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83);

(d)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

(e)   the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82);

(f)   the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or

(g)   the full-length-coding sequence of the cDNA deposited under ATCC accession number 209621.

Claim 28: The isolated nucleic acid of Claim 27 comprising a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO: 83).

Claim 29: The isolated nucleic acid of Claim 27 comprising a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide.

Claim 30: The isolated nucleic acid of Claim 27 comprising a nucleic acid sequence

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encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83).

Claim 31: The isolated nucleic acid of Claim 27 comprising a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide.

Claim 32: The isolated nucleic acid sequence of Claim 27 comprising the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82).

Claim 33: The isolated nucleic acid sequence of Claim 27 comprising the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82).

Claim 34: The isolated nucleic acid sequence of Claim 27 comprising the full-length coding sequence of the cDNA deposited under ATCC accession number 209621.

Claim 35: An isolated nucleic acid that hybridizes under high stringency conditions to:

    (a)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO:83);

    (b)   a nucleic acid sequence encoding the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

    (c)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83);

    (d)   a nucleic acid sequence encoding the extracellular domain of the polypeptide shown in Figure 32 (SEQ ID NO: 83), lacking its associated signal peptide;

    (e)   the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82);

    (f)   the full-length coding sequence of the nucleic acid sequence shown in Figure 31 (SEQ ID NO:82); or

    (g)   the full-length-coding sequence of the cDNA deposited under ATCC accession number 209621.

Claim 36: The isolated nucleic acid of Claim 35, wherein said hybridization occurs under high stringency conditions selected from the group consisting of:

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50% formamide, 5 x SSC (0.75 M sodium chloride, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% sodium dodecyl sulphate, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (0.75 M sodium chloride, 0.075 M sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC (0.75 M sodium chloride, 0.075 N sodium citrate) containing EDTA at 55°C.

Claim 37: The isolated nucleic acid of Claim 35 which is at least 10 nucleotides in length.

Claim 38: A vector comprising the nucleic acid of Claim 25.

Claim 39: The vector of Claim 38, wherein said nucleic acid is operably linked to control sequences recognized by a host cell transformed with the vector.

Claim 40: A host cell comprising the vector of Claim 38.

Claim 41: The host cell of Claim 40, wherein said cell is a CHO cell, an *E. coli* or a yeast cell.

Immunosuppressive therapy

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Although Cyclosporin A has improved transplant outcome, its use has serious limitations due to its narrow therapeutic window. New approaches to broaden this window exploit alternative drug formulations, pharmacokinetic profiling and new immunosuppressive agents, such as Rapamycin and Brequinar, which act in a synergistic fashion. There is no evidence to suggest that the pharmacological alternative to Cyclosporin A, FK-506, displays a broader therapeutic window, although it may be tenfold more potent. Similarly, despite the specificity of the IgG2a mouse anti-human CD3 monoclonal antibody, it displays a significant range of clinical side effects, delayed therapeutic action and frequently stimulates generation of human anti-mouse monoclonal antibodies. Recent advances in monoclonal antibody technology seek not only to produce antibodies against determinants involved in alloactivation, but also to 'humanize' the antibodies for reduced side effects. The availability of this array of potential agents highlights the need to develop guidelines for clinical trial methodologies to address the unique needs and demands of organ transplantation.

Introduction

After thirty years of vigorous but relatively unproductive research, the field of immunosuppressive drugs awakened following the approval of two agents that, in contrast to the non-selective drugs Azathioprine (Aza) and corticosteroids, display relatively specific actions on T cells. One of these, the fungal undecapeptide Cyclosporin A (CsA), not only improved clinical outcomes and broadened the clinical settings in which transplants were successful, but also provided a unique tool for dissecting activation mechanisms leading to lymphokine synthesis. Subsequent approval of the other agent, the IgG2a mouse monoclonal antibody (mAb) OKT-3, heralded the use of reagents that bind selective T-cell surface markers to modulate the immune response. The past decade has witnessed striking progress in the development of new pharmacological agents (Fig. 1). One group inhibits lymphokine biosynthesis, FK-506 or signal transduction, Rapamycin (RAPA). A second group is the nucleotide synthesis inhibitors; Mizorbine [1] and RS61443, a morpholinoethyl-ester analog of mycophenolic acid (MPA) [2••], block purine salvage pathways with the generation of guanosine monophosphate, and the quinoline carboxylic acid Brequinar (BQR) blocks the *de novo* synthesis of pyrimidines [3••]. A third group,

new mAbs, recognizes specific surface epitopes on T cells and antigen-presenting cells (Fig. 2). Immunosuppressive activity has been documented [4–9] with several mAbs that bind various determinants as shown in Table 1. Prolonged graft survival has also been achieved with antibodies, or preferably their $F(ab')_2$ fragments, directed towards class I [10] or class II MHC antigens. A refinement of mAb technology is the production of immunotoxins. Ricin α-chain toxin linked to mouse anti-human CD5 IgG1 mAb has been used by Haverty (personal communication) to treat steroid-resistant graft versus host disease in human bone marrow transplantation. This array of new agents proffers an unprecedented opportunity to design effective, yet minimally toxic, regimens to improve the outcome of transplantation in man.

## Limitations of existing immunosuppressive regimens

Currently, clinical regimens are based upon the use of CsA, the immunosuppressant benefits of which are seriously limited by side effects. In attempts to augment its efficacy, the corticosteroid Prednisone (Pred), Aza,

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Abbreviations

ALG—anti-lymphocyte sera; Aza—Azathioprine; BQR—Brequinar; CMV—cytomegalovirus; CsA—Cyclosporin A; CTL—cytotoxic T lymphocyte; DTH—delayed type hypersensitivity; ICAM—intercellular adhesion molecule; IL—interleukin; LFA—lymphocyte function-associated antigen; mAb—monoclonal antibody; MHC—major histocompatibility complex; MPA—mycophenolic acid; MZB—mizorbine; NF-AT—nuclear factor of activated T cells; Pred—Prednisone; RAPA—Rapamycin; TCR—T-cell receptor; Th—T-helper.
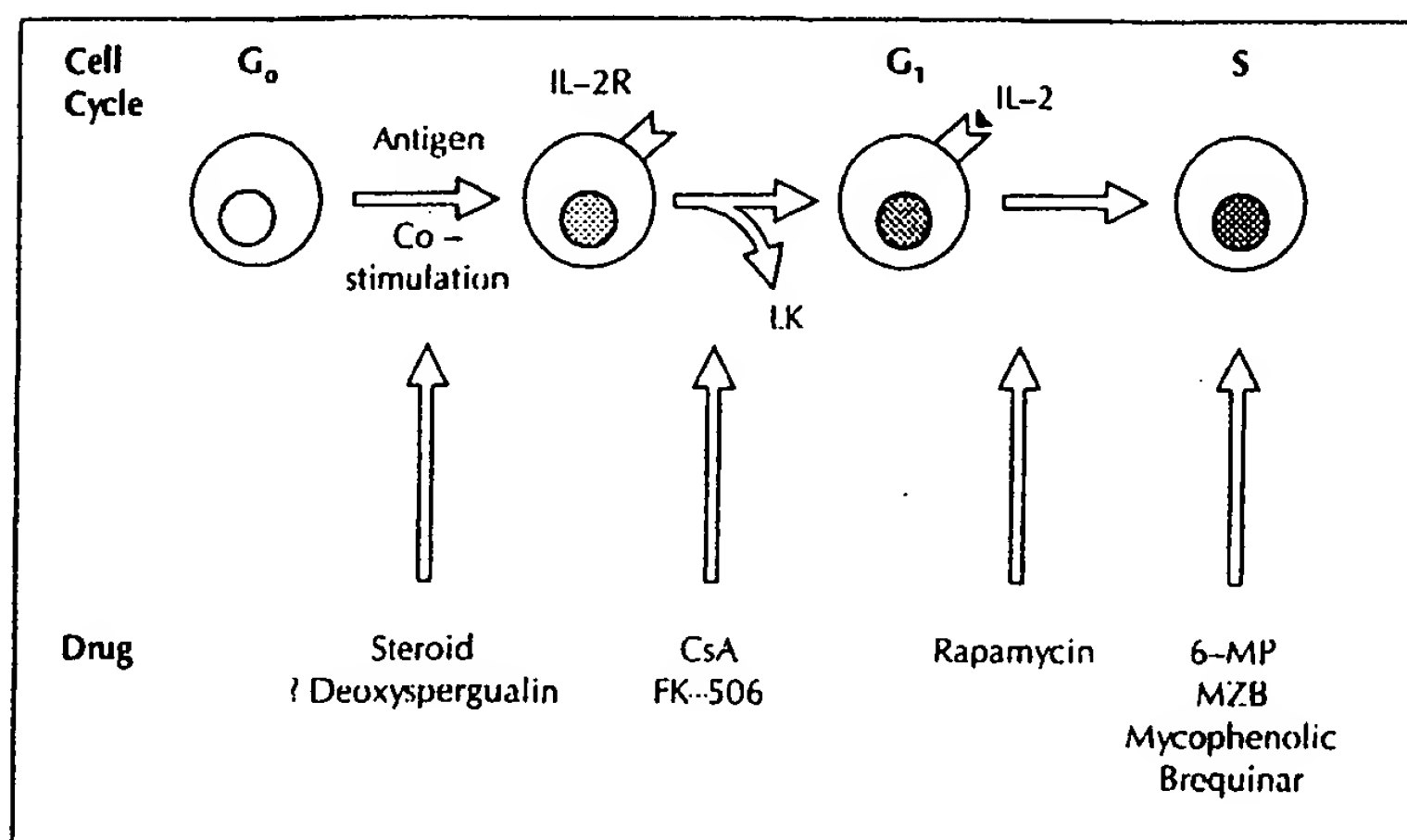
Fig. 1. Classification of immunosuppressive drugs based upon their site of action in the cell cycle. In the first group, corticosteroid, and possibly deoxyspergualin, inhibit antigen- presenting cells. In the second group, Cyclosporin A (CsA) and FK-506 inhibit lymphokine (LK) biosynthesis during the G_0 phase and Rapamycin inhibits signal transduction during the G_1 phase. In the third group (the nucleoside synthesis inhibitors), Mizorbine (MZB) and RS61443 (a morpholinoethyl-ether analog of mycophenolic acid, MPA) inhibit purine synthesis pathways leading to the generation of guanosine monophosphate, whereas Brequinar (quinoline carboxylic acid) inhibits the de novo synthesis of pyrimidines. IL, interleukin; R, receptor.
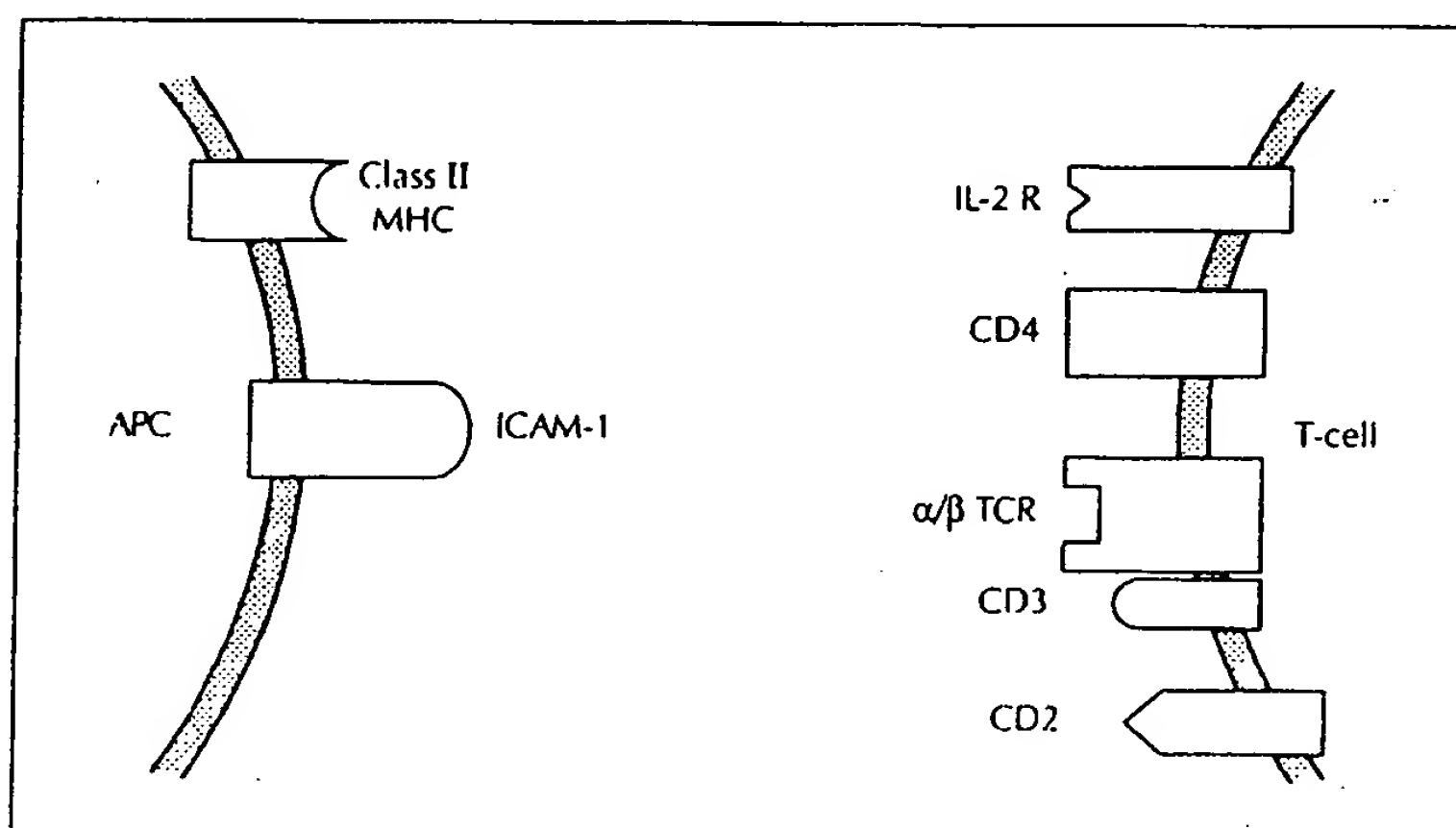
Fig. 2. Epitope targets of monoclonal antibodies. ICAM, intercellular adhesion molecule; IL, interleukin; MHC, major histocompatibility complex; R, receptor; TCR, T-cell receptor.

Table 1. Determinants recognized by immunosuppressive monoclonal antibodies

Monoclonal antibody	Determinant	Reference
IgM T1089.1.A-31	Determinants common to α/β	[4]
IgG2b BMA 031	chains of all human	[5]
	T-cell receptors	
OKT4A	CD4	[6]
SDZ CHH 380	CD7	[7]
33B3.1	α chain or α/β complex	[8]
	of interleukin-2 receptor	
BIRR-1	Intercellular adhesion	[9]
	molecule-1 (or CD54)	
25.3	Lymphocyte function-associated antigen-1	

equine or rabbit polyclonal anti-lymphocyte sera (ALG) and/or mouse OKT-3 mAbs have been combined in empirical regimens that are often tailored to individual patients. To address one limitation of CsA, nephro- and/or hepato-toxicity during the initial post-transplant phase of induction immunosuppressive therapy, which delays allograft and patient recovery, pharmacokinetic control

programs have been used either to pre-select CsA doses [11] or to combine ALG or OKT-3 with Aza/Pred in order to delay treatment with CsA. However, immediate, rather than delayed, administration of CsA to patients displaying good initial renal function avoids the disadvantages of ALG/OKT-3 induction, namely an increased risk of cytomegalovirus (CMV) infection, additional expense and/or delayed hospital discharge awaiting satisfactory CsA levels after treatment with CsA. However, patients at extraordinarily high immunological risk, due to rejection of previous allografts within three months, or with marginally-functioning organs may preferably be treated with ALG or OKT-3 induction as a possible means to delay the onset of their first rejection episodes. Two possible alternatives for induction therapy are the CsA analogs that may display reduced nephrotoxicity, namely Cyclosporin G [12], which substitutes norvaline at position 2, and IMM-125 with a hydrophilic substituent at position 9.

To date, no large randomized study has shown that the induction regimen alters the clinical outcome; rather, a small cohort reported by Belitsky et al. [13] showed no difference between ALG versus initial CsA therapy, the two options for induction therapy. There may be several reasons for this. Firstly, the polyclonal reagents, such as

rabbit or equine Minnesota ALG and anti-thymocyte globulin, regardless of their source, opsonize T cells, leading to their removal from the circulation. This depletion obviates T-cell mediated attack on the allograft. Among other factors that must be considered is that the central intravenous lines required for polyclonal administration may be accidentally contaminated, producing septicemia. Further, there are no indices of the efficacy of the polyclonal sera. Peripheral blood T-cell numbers above the target range of 50–150/ml tend only to be useful reflections of the appearance of human host anti-equine antibodies, not immunological resistance to therapy. The other induction therapy using OKT-3, which covers and/or modulates CD3 epitopes on the T-cell surface, offers the advantages of both peripheral intravenous administration and readily available fluorescence-activated cell sorting (FACS) tests for antibody efficacy. The clinician monitors patient peripheral blood lymphocyte T-cell epitopes for (a) cells with exposed CD3 epitopes that were not bound by OKT-3 *in vivo* by their capacity to bind fluorescenated OKT-3 *in vitro*, (b) the total number of circulating T cells with fluorescenated anti-CD2, a pan T-cell marker, and (c) the proportion of OKT-3-coated cells detected with a goat anti-mouse IgG reagent. A satisfactory therapeutic effect is observed when the patient has $<25\%$ OKT-3$^+$ cells *in vitro* and 60–75% CD2$^+$T cells, about 40% of which are coated with mouse IgG which binds to OKT-3. While OKT-3 represents an advance in immunosuppressive therapy, it has several serious limitations: (a) severe first-dose reactions, including chills, fever, myalgias and, in the worst cases, pulmonary oedema apparently due to lymphokine release (particularly tumor necrosis factor and interleukin (IL)-2; (b) longer-term adverse effects such as aseptic meningitis; (c) a delay in the therapeutic effect for as long as 7 days after initiation of treatment; (d) induction of human anti-mouse antibodies, generally of the anti-idiotype variety, but not uncommonly of broader reactivity; (e) a frequent incidence of rebound re-rejection episodes upon completion of the therapeutic course; and (f) a tendency toward CMV infections in 40% of treated patients. In addition, both polyclonal and mAb reagents may produce excessive immunosuppression, resulting in increased incidences of CMV infection and/or of lymphomas and other neoplasms, as well as allograft thrombosis. Thus, selection of a CsA versus an antibody induction regimen must balance the risks of nephrotoxicity versus that of excessive immunosuppression.

The use of CsA has reduced the risk of acute rejection, but a rational approach to CsA administration is confused by the tremendous variability between individuals in drug pharmacokinetics and pharmacodynamics [14], which, in turn, generates a fear of irreversible renal injury in case the CsA dose is excessive. Three approaches have been used to address this problem: (a) combining reduced CsA doses with subtherapeutic amounts of Aza [15]; (b) monitoring the parent compound CsA based upon its trough concentration prior to the next drug dose [16]; and (c) adjusting CsA doses prospectively based upon average concentrations calculated from serial measurements of the area under the concentration-time curve

[11]. Since drug absorption presents the greatest variability in pharmacokinetics, attempts have been made to increase CsA bioavailability by co-administration of Vitamin E [17]. In addition, the manufacturer has produced a new micro-emulsion formulation that increases the bioavailability by twofold above that of the existing oral solution or capsule preparation, which show equivalent bioavailability in studies comparing both formulations [18]. The critical issue seems to be the drug concentration in the allograft. While direct intra-arterial infusion has been used for experimental models of renal or cardiac allografts, good drug uptake in man can be achieved by presenting CsA as an aerosol in absolute ethanol when it has a mean particle diameter of 1.2 microns [19].

Optimal use of CsA demands the measurement of drug concentrations/activities at the level of its lymphocyte receptor or target signal transduction molecule(s), which may be calcineurin (an enzyme that may be involved in a common step associated with T-cell and IgE receptor signaling pathways) [20••] or the nuclear factor of activated T-cells (NF-AT). However, a major limitation may be the failure of CsA to inhibit lymphocyte activation via the CD28 surface marker [21], an important co-stimulatory pathway that together with T-cell receptor (TCR) stimulation blocks induction of anergy in T-cell clones [22]. Fortunately, rejection episodes under CsA prophylactic therapy tend to be readily reversed by corticosteroid therapy, and the majority of steroid-resistant episodes are overcome with polyclonal ALG and/or OKT-3 therapy. Corticosteroids are believed to represent the Achille's heel of transplantation because of the wide distribution and pleiotropic effects of the glucocorticoid receptor superfamily found in the cytoplasm. These are DNA-binding dimeric transcription factors with a zinc finger structure that recognize enhancer (or negative regulator) elements bearing the GRE motif (GTACAnnnT-GTTCT, where n = any nucleotide). One important negative regulatory element is the AP-1 binding site, normally the focus for fos-jun heterodimers [23]. An alternative approach to the reduction of IL-1β generation, an action typical of corticosteroids, is to inhibit the enzyme that cleaves the inactive 31 kD precursor between Asp^{116} and Ala^{117} to release the 153 carboxyl-terminal amino acids that constitute IL-1β [24]. Another immunosuppressive effect may be achieved by the upregulation of the synthesis of transforming growth factor-β by steroids [25]. Withdrawal of steroid treatment months to years after the transplant may be successful in patients who did not reject the transplant [26•].

Preliminary data suggest that a ten-day course of the IgM mouse anti-human α/β TCR mAb T10B9.1A-31 [4], but not BMA031 (C Groth, personal communication), not only produces equivalent therapeutic effects to those of OKT-3, but is less toxic in terms of incidence of fever and neurological and respiratory symptoms, as well as of subsequent infections. Furthermore, T10B9 therapy is not associated with as great a rise in serum creatinine during treatment as OKT-3, suggesting a more rapid attenuation of the allo-immune response. However, the repeated use of xenogeneic antibodies during the induc-

tion phase as well as for anti-rejection therapy may be complicated by the development of neutralizing human anti-mouse antibodies.

A major goal of maintenance immunosuppressive therapy is prophylaxis against chronic rejection. To date, not only has CsA/Pred therapy failed to reduce the incidence of this complication from the 8–10% level observed under the Aza/Pred combination, but there is no way to determine if the failure is due to its inherently modest inhibition of B-cell responses or to physicians' tendency to limit CsA therapy to minimal, possibly ineffective, doses in order to mitigate a renal injury. Thus, despite the improvement in initial graft survival, transplants continue to be lost in the longer term, with half-lives of about seven years for cardiac and 11.5 years for renal transplant in humans. A recent study of the effects of immunosuppressive drugs on coronary vascular disease in heterotopic rat cardiac allografts suggests that RAPA particularly, CsA to a lesser extent, but definitely not FK-506, inhibit pathological endothelial and smooth muscle lesions in arteries and arterioles, which seem to be the critical lesions in the progression of chronic rejection [27].

New pharmacological agents

Both the macrolide FK-506 and the undecapeptide CsA interrupt lymphokine synthesis by inhibiting generation of the Ca^{2+} dependent regulatory proteins NF-AT, NFIL-2A, NFIL-2B, and NF-$\times\beta$, but not c-fos, which is necessary for IL-2 generation. Presumably, both drugs also affect serine protease gene transcripts, an excellent marker of rejection [28]. The inhibition of cytotoxic T lymphocytes (CTLs), even in the presence of optimal amounts of IL-2, is a prominent effect of CsA [29] and, apparently, FK-506. Despite the assumption that CsA and FK-506 produce similar inhibitory effects, at least three differences have been observed: first, FK-506 displays a flatter inhibition curve than CsA with a wider discrepancy in potency at the 50% inhibition than at the 95% inhibition level; secondly, CsA leads to the generation of suppressive T cells, whereas FK-506 does not; and thirdly, although both drugs inhibit $CD4^+$ T helper (Th) lymphocytes, which secrete IL-2, only CsA (and not FK-506) permits priming of $CD8^+CTLs$ [30]. Furthermore, Bretscher and Havele [31] suggest that CsA switches the immune response to the graft from a delayed type hypersensitivity (DTH) response to an IgG response by inhibiting the Th1 subset with the emergence of the Th2 subset, which actively induces IgG via IL-4 generation and inhibits Th1 cells and DTH via IL-10. Both CsA and FK-506 spare transcription of the down-regulatory lymphokine IL-10. While CsA inhibits transcription of IL-6, this factor is not affected by FK-506.

The coming year should witness publication of a vast array of randomized trials comparing the clinical outcome of liver and renal transplants in patients treated with FK-506 versus CsA. So far, a preliminary non-randomized study of liver recipients showed that FK-506 therapy displays greater neurotoxicity, equivalent nephrotoxicity,

but, possibly, less hypertension than does CsA therapy [32••]. A further claim that corticosteroids do not have to be used with FK-506 cannot be assessed due to two factors: firstly, the protocol stipulated higher Pred doses in the CsA cohort than those used with FK-506; and secondly, to date, there is no pharmacokinetic analysis of Pred concentrations in CsA versus FK-506 treatment groups in order to exclude a drug interaction. Additionally, the extremely poor results in the initial study, wherein allegedly CsA-resistant patients were converted to treatment with FK-506, actually reflected antagonism between the two drugs caused by (a) an adverse immunological interaction between the two agents that apparently have similar mechanisms of action [33] and (b) competitive pharmacokinetic interactions. Although FK-506 has not yet been shown to achieve clinical results even equivalent to those of CsA, eventual definition of its relative therapeutic window will depend upon Phase II studies to select well-tolerated drug doses for randomized trials versus CsA therapy.

When a second agent, RS61443, was added in doses of 2 500–3 500 mg per day to a CsA/Pred regimen, it seemed to reduce the incidence of acute rejection episodes. However, these high doses are likely to produce toxicity, particularly leukopenia and gastrointestinal complaints [2••]. Randomized placebo-controlled trials are underway to assess the efficacy of RS61443 versus Aza added to a CsA/Pred regimen. Other studies are examining the impact of a fourth agent, deoxyspergualin, to potentiate an ALG/Aza/Pred/CsA induction protocol.

The studies that claimed Aza displays pharmacological synergism with CsA failed to utilize rigorous experimental design or data analysis [34]. For instance, both *in vitro* analyses [35] and clinical results demonstrate that Aza acts in an additive manner rather than synergistically with CsA [36]. Similarly, *in vitro* analyses suggest that RS61443 [37], mizorbine [37], and thalidomide [38] also act in an additive manner with CsA. Although initial data suggested that BQR potentiates the effect of CsA [3••], recent experiments document true synergism [37]. However, CsA/RAPA combinations show the most impressive degree of synergy both *in vitro* and *in vivo* [39]. Once Phase I toxicity trials have been completed, it will be possible to assess whether BQR or RAPA displays the synergistic effects with CsA in human transplantation that are evident in rodents and large animal models.

New monoclonal antibody reagents

Second generation mAbs are being designed to avoid the severe systemic reactions due to lymphokine release that follow initial doses of OKT-3. For example, the IgG2b anti-human α/β TCR mAb BMA 031 used for induction therapy (three 50 mg doses administered on alternate days) delays the onset of first rejection episodes and probably improves one-year graft survival (R Knight and BD Kahan, unpublished data). Similar benefits have been reported with mouse and rat mAbs produced against the activation-induced α-chain, or to new epitopes resulting

from the formation of the $\alpha\beta$ complex, of the IL-2 receptor [8].

However, treatment with these antibodies leads to a high incidence of human anti-mouse antibodies, which may attenuate the immunosuppressive effects. Recent work has explored approaches to construct either (a) 'chimeric' antibodies bearing human Fc segments joined to mouse $F(ab')_2$ fragments, or (b) 'humanized' mAbs with mouse idiotypes inserted onto human IgG isotypes (Fig.3). Chimeric antibodies combine the variable regions of mouse antibodies with human antibody constant regions and, therefore, present fewer foreign amino acid sequences to the host. However, one-third of the structure is still of mouse origin. Furthermore, a clinical trial using a chimeric anti-CD7 mAb not only failed to achieve a superior level of immunosuppression induction, but also increased the incidence of vascular thromboses [7]. The latter effect may have been related to the adhesion of Fc receptors on platelets and polymorphonuclear leukocytes to the human Fc regions, bound to endothelium via mouse epitopes. On the other hand, 'humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen combining site, with human variable region frameworks and constant regions. Due to the reduced affinity of 'humanized' antibodies for antigen epitopes, Co et al [40•] recommended two innovations: firstly, selection of a human framework that is as homologous to the original mouse antibody as possible; and secondly, insertion of key residues from the mouse model into the construct in order to achieve a molecular conformation that is similar to the native idiotype. The beneficial effects of chimeric and 'humanized' variants of mouse mAbs will be clarified only by randomized clinical trials.

Two alternative approaches seek to utilize mAbs directed against donor MHC antigens or against co-receptor molecules. In a study of non-human primates,

OKT-4A IgG2A mAbs, which react with the CD4 co-receptor on Th cells, provoked fewer side effects than OKT-3 [6]. In an initial clinical trial of OKT-4A induction therapy (0.2 mg/kg/day), all six patients suffered rejections. These rejections were reversible, but left residual areas of dead tissue resulting from an obstruction of the blood supply in half the renal allografts. Unfortunately, OKT-4A also generated a strong human anti-mouse antibody response (J Barry, personal communication). Experimental animal models are currently being used to determine if antibody efficacy is related to T-cell deletion and is potentiated by simultaneous treatment with an anti-CD8 mAb. On the one hand, Fathman and colleagues [41] found that depleting anti-CD4 mAbs produced prolonged allounresponsiveness toward allogeneic pancreatic islets, an effect that was moderated by simultaneous treatment with anti CD8 + mAbs, suggesting the role of a regulator CD8 + cell. On the other hand, Waldmann and colleagues [42] induced tolerance toward mouse heart transplants where the donor and recipient were not matched at MHC level using an anti-CD4 mAb that not only did not deplete T cells but also was potentiated by simultaneous administration of an anti-CD8 mAb.

A second approach to co-receptor molecules is based on the interaction of lymphocyte function-associated antigen (LFA)-1 on CTLs with the intercellular adhesion molecule (ICAM)-1 or monocytes. Expression of ICAM-1 is up-regulated following lymphokine release, which occurs during acute allograft rejection but not during other pathological events in the kidney [43••]. Prophylactic and therapeutic administrations of a mAb directed against the high molecular weight α-chain of human ICAM-1 alone delayed both the onset and progression of rejection episodes in primate renal allograft models. Using mouse mAbs directed against LFA-1, Stoppa et al [44] reversed steroid resistant acute graft versus host reactions in man. Indeed, the combination of anti-ICAM-1 and anti-LFA-1 mAbs produced allo-tolerance in mice that were not com-

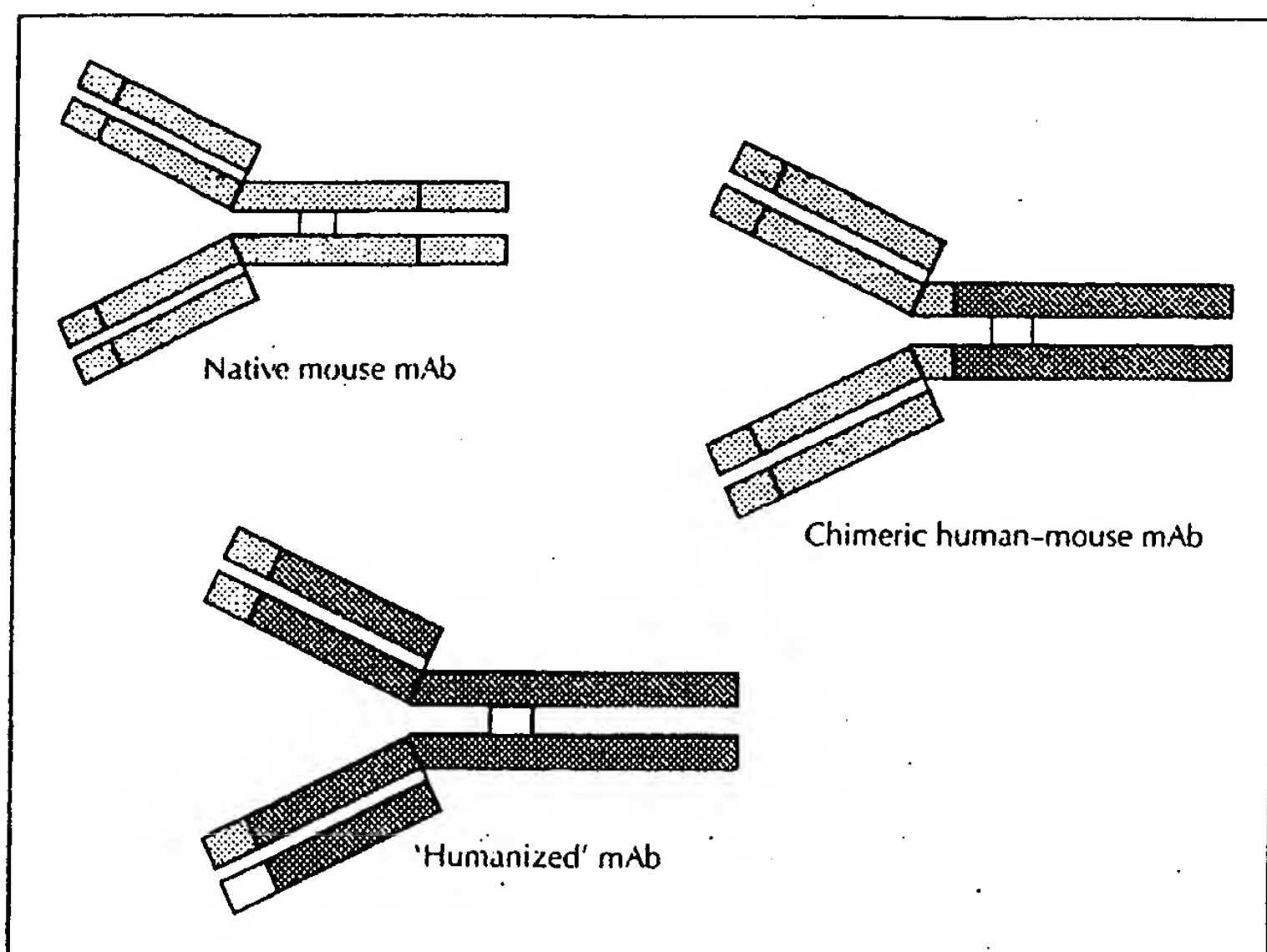Native mouse mAb

Chimeric human–mouse mAb

'Humanized' mAb

Fig. 3. Types of monoclonal antibody. Chimeric antibodies combine the variable regions of mouse antibodies with human constant regions and, therefore, present fewer foreign amino acid sequences to the host. 'Humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen binding site, with human variable region frameworks and constant regions.

patible at the MHC level [45••]. These promising results in animal models using mAbs directed against T-cell and monocyte co-receptors await confirmation in controlled clinical trials.

While clinical interventions to date have focused on using mAbs directed towards surface epitopes important for the afferent limb of the allo-immune response, there is increasing evidence that anti-idiotypic antibodies, either exogenously introduced or endogenously, spontaneously generated, may regulate the induction of allo-immune responses. A recent study performed by Snider [46] suggested that immunization of hosts with antigen–antibody complexes confers a bias in the epitope, resulting in a less efficient antibody response that shows anti-idiotypic properties. This approach represents a particularly fertile ground for clinical exploration.

Cytokine receptor analogs and antagonists

A new group of immunosuppressive agents are the cytokine receptor antagonists. The discovery and initial testing of an IL-1 receptor antagonist has been reviewed by Arend [47]. IgG-stimulated human monocytes naturally produce IL-1 receptor antagonist, a heterogenous array of glycoproteins of 15–25 kD, depending upon their degree of glycosylation. IL-1 receptor antagonist binds type I, but not type II, IL-1 receptors without activating cells and with considerably less avidity than native IL-1α and IL-1. Type I IL-1 receptors are present on Th2 cells and fibroblasts; Type II IL-1 receptors are present on B cells, neutrophils, and macrophages. Although therapeutic trials of IL-1 receptor antagonist in rheumatoid arthritis and septic shock suggest some beneficial effects, Faherty et al. [48] failed to observe that IL-1 receptor antagonist inhibited induction of CTLs, cutaneous DTH, or T-cell dependent humoral antibody responses. They also found that administration of a mAb to type II IL-1 receptor (35F5) was ineffective. Fanslow et al. [49•] recently extended their previous studies, which used constructs of the extra-membranous portion of the IL-1 receptor, by using similar constructs of the IL-4 receptor. In the initial studies, they prolonged heterotopic pinnal, neonatal mouse heart allograft survival, but failed to prevent allo-sensitization, as documented by a rapid, secondary-type proliferative response upon in vitro one-way mixed lymphocyte reactions. In their recent studies, constructs of IL-4 receptor alone, or in combination with rat anti-mouse IL-4 receptor mAb, induced modest prolongation of heart-allo-explants.

Immunosuppressive drug trials

Of the numerous obstacles currently hindering the development of efficacious immunosuppressive regimens, the lack of methodology for clinical transplantation trials is of particular importance. To date, no series of Phase I and II toxicity and dose-finding trials has been conducted in

order to establish a foundation for clinical investigation. The introduction of Aza, steroids, and CsA, as well as the preliminary trials of FK-506, have relied upon empirical approaches. Important obstacles to comprehensive trials include the relatively small numbers of transplant cases, the use of unrefined end-points such as graft and patient survival, and the lack of well-established criteria for the diagnosis and grading of rejection episodes, deficits that obfuscate the use of this event as an intermediate end-point. In addition, no in vitro immune assay predicts or correlates with in vivo immunosuppressive efficacy; hence, there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from in vitro systems to in vivo conditions.

Since present results with CsA-based regimens yield excellent graft survivals, extremely large numbers of patients must be entered into clinical trials to document improved efficacy of a new agent. Even more extensive efforts will be needed to exclude the possibility that the results with the new agent are not actually worse than those obtained with the existent CsA regimen. In light of the presently high success rates, the benefits of any new regimen must be based upon both the potency and the mitigation of side effects, as assessed by quantitative parameters, including glomerular filtration rates. The practice of clinical research in transplantation must proceed to develop principles of rigorous study design and precise analytical tools in order to most expeditiously evaluate the available array of new immunosuppressants described in this review.

Acknowledgements

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest

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INTERLEUKIN-12 (IL-12)-DRIVEN ALLOIMMUNE RESPONSES IN VITRO AND IN VIVO

REQUIREMENT FOR β1 SUBUNIT OF THE IL-12 RECEPTOR[1]

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Background. Interleukin-12 (IL-12) mediates its biologic activities via binding high-affinity receptors on T and natural killer cells. Although emphasis has been placed on the requirement for IL-12Rβ2 in IL-12 bioactivity, the role of IL-12Rβ1 is less well defined. The current study evaluated the effects of exogenous IL-12 on alloantigen-specific immune responses and determined the requirement for IL-12Rβ1 in IL-12-mediated alloimmunity.

Methods. The mouse heterotopic cardiac transplant model was employed to evaluate the effects of IL-12 on alloantigen-specific immune responses in vivo. In addition, IFN-γ production in mixed lymphocyte cultures (MLC) supplemented with IL-12 was measured to assess the effects of IL-12 on Th1 function in vitro. Mice deficient in IL-12Rβ1 (IL-12Rβ1$^{-/-}$) were used to determine the requirement for this receptor component in IL-12-driven alloimmune responses.

Results. Addition of IL-12 to MLC consisting of wild-type splenocytes enhanced alloantigen-specific proliferative responses and Th1 development. In contrast, IL-12 did not alter these in vitro immune parameters in IL-12Rβ1$^{-/-}$ MLC. Treatment of wild-type cardiac allograft recipients with IL-12 resulted in high concentrations of serum interferon-γ (IFN-γ) and a 10-fold increase in IFN-γ production by recipient splenocytes after restimulation in vitro. However, this fulminate Th1 response did not accelerate allograft rejection. Importantly, IL-12 had no effect on serum IFN-γ or in vivo priming of Th1 in IL-12Rβ1$^{-/-}$ recipients. Finally, administration of IL-12 to WT allograft recipients resulted in a bimodal alloantibody response: antibody production was suppressed at high doses of IL-12, and enhanced at lower doses.

Conclusions. IL-12 markedly enhances alloantigen-specific immune function; however, these exaggerated Th1-driven responses do not culminate in accelerated allograft rejection. Further, these data indicate that IL-12Rβ1 is essential for the enhancement of both in

vitro and in vivo alloimmune responses by exogenous IL-12.

It is well established that interleukin-12 (IL-12*) is a critical cytokine involved in the regulation of Th1- and Th2-mediated immune responses in several experimental models (reviewed in *1* and *2*). IL-12 has direct stimulatory and inhibitory effects on Th1 and Th2, respectively (*3–6*). Further, this cytokine promotes Th1 and inhibits Th2 development indirectly by inducing interferon-γ (IFN-γ) production by activated T cells and natural killer cells (*7–12*). Th1 have been accepted as key regulators of allograft rejection, in that this cell type promotes both delayed-type hypersensitivity and cytotoxic T lymphocyte responses, which are believed to be the principle terminal effector mechanisms of acute allograft rejection (*13, 14*). An understanding of the role of IL-12 in graft rejection is just emerging. For example, IL-12 clearly augments alloreactive Th1 development in vitro (*15*). However, the presence of IL-12 is not mandatory for the development of acute cardiac allograft rejection (*15, 16*). Hence, an important question is whether enhanced Th1 function alters the rejection response. Given the IL-12/Th1 dogma, one would predict that IL-12 would augment alloreactive Th1 function, resulting in accelerated allograft rejection. The present study therefore was designed to test the hypothesis that IL-12-driven Th1 responses would exacerbate cardiac allograft rejection.

IL-12 mediates its biologic effects by interacting with a high-affinity receptor, which consists of at least two cloned components, IL-12Rβ1 and IL-12Rβ2 (*17–19*). IL-12Rβ1 interacts with the p40 subunit of IL-12, whereas the p35 subunit of IL-12 is believed to bind to IL-12Rβ2 (*19, 20*). Emphasis has been placed on the necessity for IL-12Rβ2 in IL-12 signaling (*21, 22*). However, by utilizing IL-12Rβ1 knockout mice (IL-12Rβ1$^{-/-}$), Wu et al. (*23*) recently reported that the β1 subunit of IL-12R is essential for IL-12-driven proliferation and IFN-γ production by mitogen-activated blasts, natural killer cell lytic activity, and IFN-γ production in response to endotoxin. We have reported that the p40 subunit of IL-12 stimulates alloreactive CD8+ Th1 development both in vitro (*24*) and in vivo (*15*). These observations suggest that

* Abbreviations: Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; H&E, hematoxylin and eosin; IFN-γ, interferon-γ; IL, interleukin IL-12Rβ1$^{-/-}$, mice deficient in β1 subunit of IL-12 receptor; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; WT, wild-type.

IL-12Rβ1 may be needed for alloreactive Th1 development, and that signaling through IL-12Rβ1 may be sufficient to mediate IL-12's biologic activity on CD8+ T cells. Hence, the present study employed IL-12Rβ1$^{-/-}$ mice to determine whether β1 subunit of IL-12R is required for IL-12-induced alloantigen-specific immune responses. To our knowledge, this study is the first to investigate the effects of IL-12 treatment on alloreactive Th1 development in vivo and to establish a mandatory role for IL-12Rβ1 in IL-12-driven alloimmune responses.

MATERIALS AND METHODS

Mice. Wild-type (WT) C57BL/6 and BALB/c mice between 6 and 12 weeks of age were obtained from Charles River Laboratories (Raleigh, NC). Generation of C57BL/6 IL-12Rβ1$^{-/-}$ mice has been described previously (23). These mice were generated on the 129/Sv background and back-crossed to C57BL/6 mice for five generations, then intercrossed to generate homozygotes.

Medium. The culture medium used in these studies was Dulbecco's minimum essential medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μM folic acid, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 2% fetal calf serum (all obtained from Life Technologies, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical, St Louis, MO).

Mitogen-driven cytokine production. To investigate the requirement for the β1 subunit of IL-12R in mitogen-stimulated IL-10 and IFN-γ production, splenocytes (2×10^6 cells/ml) isolated from naive WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were incubated for 72 hr with 1 μg/ml concanavalin A (Con A) (Sigma Chemical). Cultures were supplemented with 1 ng/ml murine recombinant IL-12 (rIL-12) (kindly provided by Dr. Maurice Gately, Hoffmann-La Roche Inc.) to assess the effect of exogenous IL-12 on Con A-stimulated cytokine production by splenocytes of WT and IL-12Rβ1-deficient mice. Resulting supernatants were harvested at 72 hr, and the concentrations of IL-10 and IFN-γ measured by enzyme-linked immunosorbent assay (ELISA).

In vitro alloimmune responses. To assess alloantigen-specific Th1 development, splenocytes (1×10^6 cells/ml) isolated from naive WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated (5000 rads) BALB/c splenocytes (1×10^6 cells/ml). Where indicated, 1 ng/ml of murine rIL-12 was added to primary mixed lymphocyte cultures (MLC) to assess the effect of exogenous IL-12 on alloantigen-driven Th1 function and to evaluate whether Th1 from IL-12Rβ1-deficient mice were responsive to IL-12 stimulation. The concentration of rIL-12 was selected from dose-response experiments in which the amount of rIL-12 needed for maximal enhancement of alloantigen-specific proliferation was 5-10 ng/ml (data not shown). Resulting cell populations were harvested, washed three times, and restimulated (at 1×10^6 cells/ml) with irradiated BALB/c stimulator cells (1×10^6 cells/ml). MLC supernatants were collected after 24 hr (IL-4 and IL-10) or 72 hr (IFN-γ), and cytokine concentrations measured by ELISA.

In addition, splenocyte proliferative response to alloantigens was determined in cultures either left unmodified or supplemented with 1 ng/ml murine rIL-12. WT or IL-12Rβ1$^{-/-}$ C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated for 5 days with irradiated BALB/c splenocytes (1×10^6 cells/ml) in 96-well U-bottom plates (Becton Dickinson, Lincoln Park, NY) in a final volume of 200 μl (done in quadruplicate). Cultures were pulsed with 0.5 μCi/well [methyl-^{3}H]thymidine (ICN, Costa Mesa, CA) for the final 8 hr of the incubation period. [methyl-^{3}H]Thymidine incorporation was assessed on a Wallac 1205 Betaplate scintillation counter (Wallac, Turku, Finland).

Heterotopic cardiac transplantation. Intact BALB/c (H2^d) hearts were anastomosed to the great vessels in the abdomens of WT or IL-12Rβ1$^{-/-}$ C57BL/6 (H2^b) mice as described by Corry et al. (25). In this model, the transplanted heart is perfused with the recipient's blood and resumes contractions until acutely rejected, which occurs in unmodified WT recipients of this strain combination in approximately 8-9 days (15, 24). Graft function was evaluated by daily abdominal palpation. Myocyte damage and intensity of graft-infiltrating cells were assessed by routine hematoxylin and eosin (H&E) staining of paraffin-embedded sections of transplanted allografts.

Experimental groups. Cardiac allograft recipients were divided into four groups: (1) recipients injected intraperitoneally with 1 mg of anti-CD8 monoclonal antibody (mAb) (hybridoma 2.43, purified by Montana ImmunoTech Inc., Bozeman, MT) on days -2 and -1 before transplantation, (2) animals given daily intraperitoneal injections of murine rIL-12 (0.1 or 1.0 μg) on days 1-6 after transplantation, (3) recipients injected with a combination of 2.43 anti-CD8 mAb plus rIL-12, and (4) unmodified (no treatment) mice, which served as controls. Depletion of CD8+ cells (<2%) was verified by flow cytometry using anti-CD8:fluorescein isothiocyanate antibody (PharMingen).

In vivo alloimmune responses. To monitor in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from allograft recipients were restimulated with irradiated BALB/c stimulator cells (1×10^6 cells/ml), and the concentration of IFN-γ was measured by ELISA. As an additional measure of the in vivo activity of IL-12 on IFN-γ production, sera IFN-γ concentrations in WT and IL-12Rβ1$^{-/-}$ cardiac allograft recipients were measured by ELISA. Further, to assess the effect of IL-12 treatment on B cell function, sera alloantibody (IgM, IgG1, and IgG2a) levels were determined (see below).

Cytokine ELISA. Experimental samples (100 μl) were added in triplicate to plates coated with 5 μg/ml rat anti-mouse IFN-γ, IL-4, or IL-10 capture antibodies (PharMingen). Standards were employed by preparing 2-fold dilutions of murine recombinant IFN-γ, IL-4, and IL-10 (PharMingen), with a starting concentration of 25, 2.5, and 10 ng/ml, respectively. After a 1-hr incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS. One hundred microliters of rat anti-mouse secondary biotinylated antibodies (1 μg/ml) (PharMingen) was then added, and plates were incubated at room temperature for 45 min. Plates were then washed three times with 0.05% Tween 20 in PBS, and 100 ml of avidin-peroxidase (Sigma Chemicals) was added. After a 30-min incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS, and 100 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma Chemical) was then added to each well. After 20 min, absorbance was determined at 405 nm by an EL 800 microplate reader (Bio-Tek Instruments, Winooski, VT). Sample cytokine concentrations were calculated from a standard curve. The sensitivity of this assay is approximately 300 pg/ml for IFN-γ, 100 pg/ml for IL-4, and 150 pg/ml for IL-10.

Sera alloantibody determination. P815 cells (H2^d) were stained for flow cytometric analysis using dilutions of sera (1:50) obtained from cardiac allograft recipients as the primary antibody, followed by fluorescein isothiocyanate-conjugated isotype-specific anti-mouse IgM, IgG1, and IgG2a secondary antibodies (The Binding Site, San Diego, CA). Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACScan.

Statistics. Statistical analyses in this study were done using a Student's *t* test performed by the program StatView 4.1.

RESULTS

Requirement for IL-12Rβ1 in T Cell Responses in Vitro

Enhancement of mitogen-driven IFN-γ and IL-10 production by IL-12 requires IL-12Rβ1. IL-12 stimulates concomitant production of IL-10 and IFN-γ by activated T cells (15, 26, 27). To determine whether β1 subunit of IL-12R is required for production of these cytokines, C57BL/6 splenocytes isolated WT or IL-12Rβ1-deficient mice were stimu-

lated with Con A for 72 hr, and supernatant cytokine concentrations were determined by ELISA. Production of the Th1 cytokine IFN-γ by Con A-stimulated splenocytes isolated from IL-12Rβ1$^{-/-}$ mice was readily detectable (Fig. 1A), although concentrations were lower than that seen in WT controls (IL-12Rβ1$^{-/-}$=1.03 ng/ml vs. WT=5.24 ng/ml). Addition of exogenous rIL-12 significantly enhanced IFN-γ production by mitogen-stimulated splenocytes obtained from WT mice (15.79 ng/ml). In contrast, IFN-γ production by splenocytes from IL-12Rβ1$^{-/-}$ mice was not altered after the addition of rIL-12 (1.54 ng/ml).

A

B
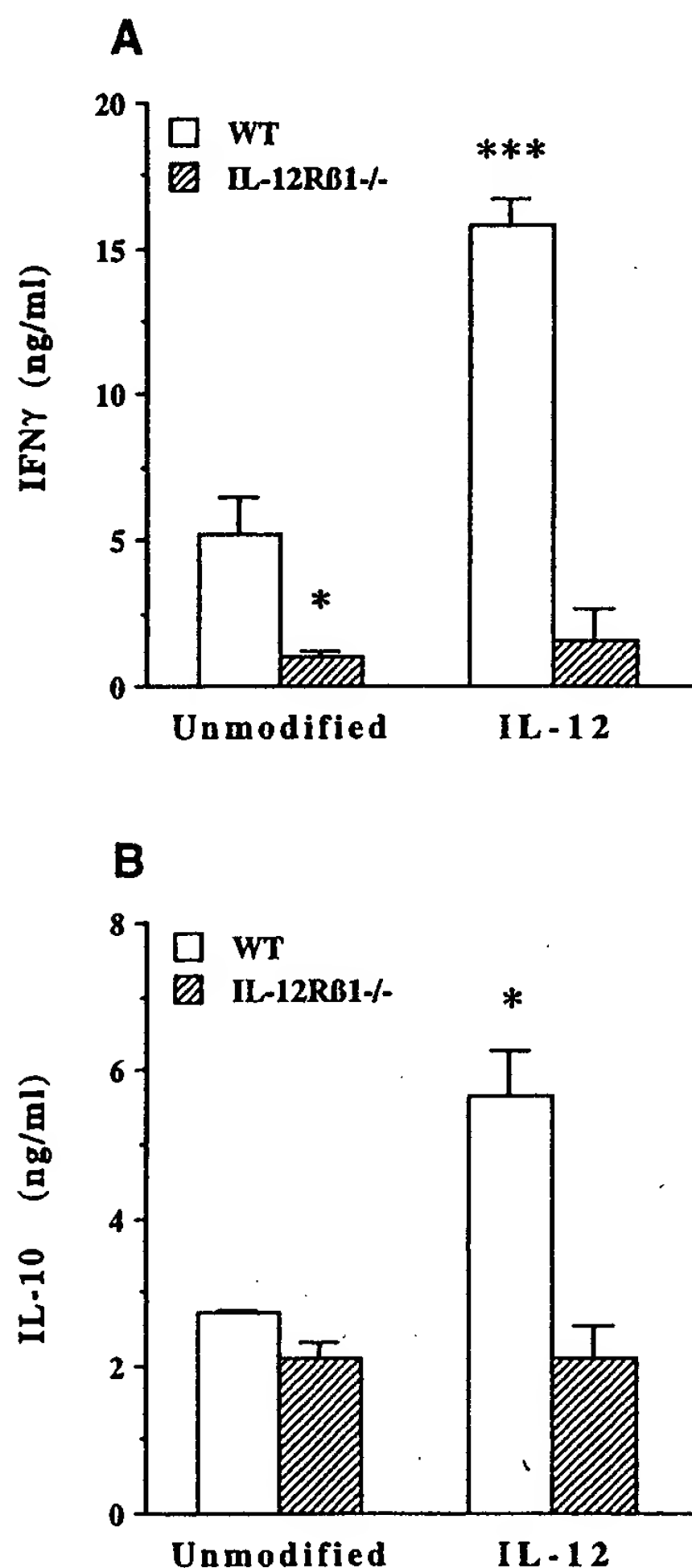
FIGURE 1. Mitogen-driven cytokine production by splenocytes isolated from IL-12Rβ1$^{-/-}$ mice. Splenocytes (2×10^6 cells/ml) obtained from WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were stimulated in vitro with 1 μg/ml Con A. Cultures were either left untreated or supplemented with murine rIL-12 (1 ng/ml). Supernatants were collected after 72 hr, and the concentrations of IFN-γ (A) and IL-10 (B) were determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples $\pm$ SD. Data are representative of three separate experiments. In panel A, *, $P<0.05$ (WT unmodified vs. IL-12Rβ1$^{-/-}$ unmodified); ***, $P<0.005$ (WT unmodified vs. WT IL-12-treated). In panel B, *, $P<0.05$ (WT unmodified vs. WT IL-12-treated).

The requirement for IL-12Rβ1 in IL-12-driven IL-10 production also was assessed. Splenocytes isolated from IL-12Rβ1-deficient mice produced similar levels of IL-10 upon Con A stimulation when compared to WT cells (Fig. 1B). rIL-12 enhanced Con A-stimulated IL-10 production by WT splenocytes (2.70 ng/ml vs. 5.65 ng/ml). However, the β1 subunit of IL-12R was required for this response, as IL-12 did not affect IL-10 secretion by mitogen-stimulated IL-12Rβ1$^{-/-}$ splenocytes.

In vitro alloreactive T helper cell development. To evaluate the requirement for IL-12Rβ1 in IL-12-driven alloantigen-specific T cell development, naive splenocytes obtained from WT or IL-12Rβ1$^{-/-}$ mice were incubated for 5 days with irradiated BALB/c splenocytes in primary MLC, which were either left unmodified or supplemented with rIL-12. Resulting cell populations were restimulated with irradiated BALB/c splenocytes in the absence of rIL-12, and in vitro IFN-γ, IL-4, and IL-10 production determined by ELISA (Table 1). Primed WT splenocytes secreted high levels of IFN-γ upon restimulation with alloantigens. Splenocytes obtained from IL-12Rβ1$^{-/-}$ mice secreted IFN-γ upon restimulation with alloantigens, albeit to a lesser degree than WT cells (WT=21.32 ng/ml vs. IL-12Rβ1$^{-/-}$=5.72 ng/ml). The decrease in alloantigen-stimulated IFN-γ production in IL-12Rβ1-deficient mice was not associated with a decrease in the cells' ability to proliferate in response to alloantigens (Fig. 2), in that [methyl-^{3}H]thymidine incorporation by alloantigen-stimulated IL-12Rβ1$^{-/-}$ splenocytes was similar to that seen by WT cells (IL-12Rβ1$^{-/-}$=13,385 cpm vs. WT=11,441 cpm). In both groups, IL-4, IL-10 (Table 1), and IL-5 (data not shown) were not detected in cultures that were not supplemented with exogenous rIL-12.

As shown in Table 1, exogenous rIL-12 markedly enhanced IFN-γ production by WT splenocytes in vitro (21.32 ng/ml vs. 215.13 ng/ml), but failed to augment IFN-γ secretion by cells obtained from IL-12Rβ1$^{-/-}$ mice (5.72 ng/ml vs. 6.80 ng/ml). Likewise, rIL-12 significantly enhanced WT splenocyte proliferation in the MLC (Fig. 2), but did not alter the proliferative ability of splenocytes isolated from IL-12Rβ1-deficient mice. Finally, the addition of exogenous rIL-12 to cultures

TABLE 1. IL-12 does not enhance alloantigen-specific Th1 development in IL-12Rβ1$^{-/-}$ mice in vitro[a]

Treatment	IFN-γ (ng/ml)	IL-4 (ng/ml)	IL-10 (ng/ml)
Unmodified			
WT	21.32±0.88	ND	ND
IL-12Rβ1$^{-/-}$	5.72±0.24**	ND	ND
IL-12 (1 ng/ml)			
WT	215.13±13.52***	ND	1.54±0.21
IL-12Rβ1$^{-/-}$	6.80±0.21	ND	ND

[a] Splenocytes (1×10^6 cells/ml) obtained from WT or IL-12Rβ1$^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated allogeneic splenocytes (1×10^6 cells/ml) in unmodified MLC or MLC supplemented with murine rIL-12 (1 ng/ml). Resulting cell populations were harvested and restimulated with alloantigens for cytokine determination. Supernatants concentrations of IFN-γ (72 hr), IL-4 (24 hr), and IL-10 (24 hr) were measured by ELISA. Results are expressed as the cytokine concentration in triplicate samples (mean $\pm$ SD). Data are representative of four separate experiments. ND indicates not detectable. **, $P<0.01$, WT unmodified vs. IL-12Rβ1$^{-/-}$ unmodified; ***, $P<0.005$, WT unmodified vs. WT IL-12-treated.
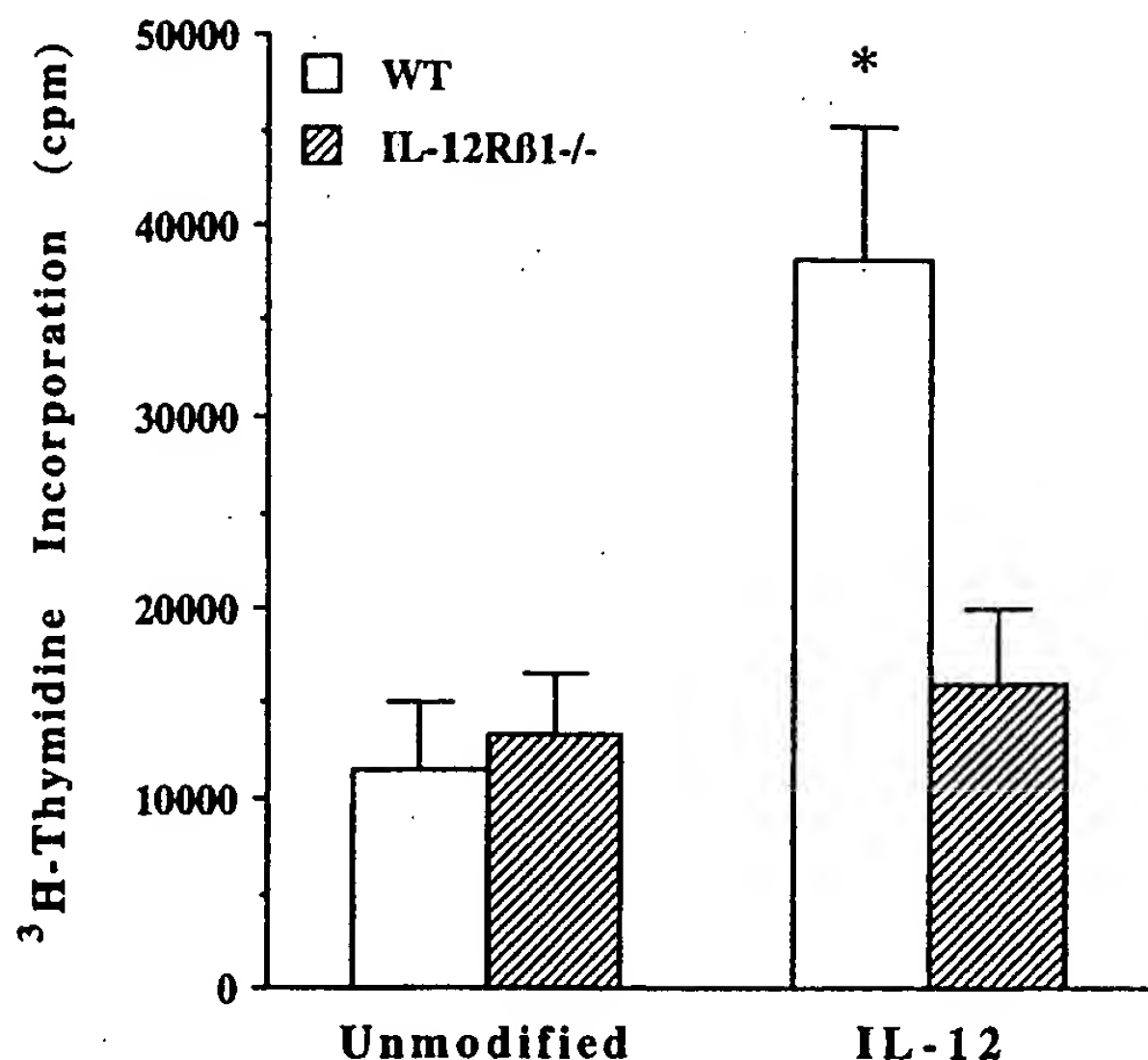
FIGURE 2. The $\beta 1$ subunit of IL-12 receptor is required for IL-12-induced stimulation of alloantigen-specific splenocyte proliferation. C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated with irradiated allogeneic BALB/c splenocytes (1×10^6 cells/ml) in 96-well microtiter plates for 5 days. Cultures were pulsed with 0.5 μCi/well [methyl-^{3}H]thymidine for the final 8 hr of the incubation period, and thymidine incorporation was determined by liquid scintillation spectrophotometry. Results are expressed as the mean cpm in quadruplicate samples ± SD. Data are representative of three separate experiments. *, $P<0.05$ (WT unmodified vs. WT IL-12-treated).

stimulated the secretion of IL-10 by alloantigen-stimulated WT splenocytes, but not IL-12R$\beta 1^{-/-}$ cells (Table 1). Collectively, these data indicate that the $\beta 1$ subunit of IL-12R is required for the enhancement of several in vitro alloimmune responses by exogenous rIL-12, including increased alloantigen-stimulated T cell proliferation, and IFN-γ and IL-10 production.

Effects of Exogenous IL-12 on Alloimmune Responses in Vivo

Enhancement of serum IFN-γ by IL-12 treatment. To monitor the in situ effects of IL-12 treatment on IFN-γ production in cardiac allograft recipients, serum IFN-γ concentrations were measured on day 7 after transplantation (Fig. 3). In both WT and IL-12R$\beta 1^{-/-}$ allograft recipients, serum IFN-γ was undetectable by ELISA on day 7 after transplantation. Treatment of WT recipients with rIL-12 markedly increased serum IFN-γ in three independent experiments; however, this treatment regimen had little effect on the concentration of serum IFN-γ in IL-12R$\beta 1^{-/-}$ allograft recipients.

Effects of IL-12 on alloantigen-specific Th1 development in vivo. Splenocytes obtained from cardiac allograft recipients were restimulated in vitro with donor alloantigens and supernatant concentrations of IFN-γ were determined by ELISA. This assay detects in vivo primed Th1, in that splenocytes from naive, nontransplanted mice produce minimal or undetectable levels of IFN-γ under these conditions (15, 16, 24). Restimulation of splenocytes from unmodified IL-12R$\beta 1^{-/-}$ allograft recipients with donor alloantigens resulted in the secretion of similar amounts of IFN-γ compared
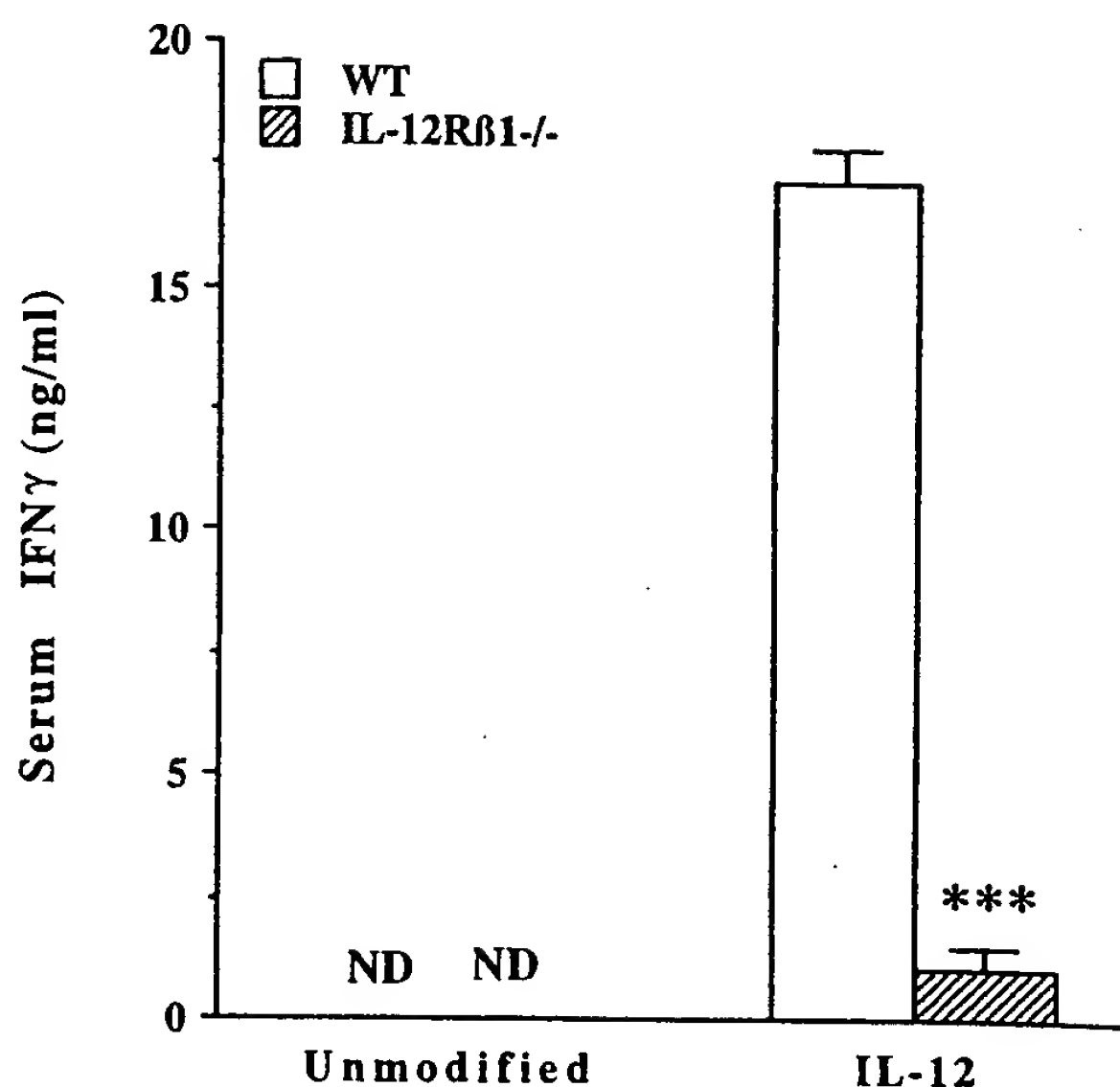
Treatment In Vivo

FIGURE 3. Treatment of WT, but not IL-12R$\beta 1^{-/-}$ allograft recipients with rIL-12 markedly increases serum IFN-γ. WT or IL-12R$\beta 1^{-/-}$ C57BL/6 mice bearing BALB/c cardiac allografts were either left untreated or given daily intraperitoneal injections of 1.0 μg of rIL-12 on days 1–6 after transplantation. On day 7, blood obtained from allograft recipients was pooled and serum collected after centrifugation. Serum IFN-γ was determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples ± SD. Data are representative of three independent experiments. ND indicates not detectable. ***, $P<0.005$ (WT IL-12-treated vs. IL-12R$\beta 1^{-/-}$ IL-12-treated).

to that seen in WT recipients (IL-12R$\beta 1^{-/-}$=7.16 ng/ml vs. WT=6.24 ng/ml) (Fig. 4). Treatment of WT recipients with IL-12 resulted in a 10-fold increase in the production of IFN-γ (59.05 ng/ml). In contrast, IFN-γ production by splenocytes obtained from IL-12R$\beta 1^{-/-}$ recipients treated with IL-12 in vivo was similar to untreated values (9.59 ng/ml), indicating that the $\beta 1$ subunit of IL-12R is required for IL-12-mediated enhancement of in vivo sensitization of IFN-γ-producing cells. Further, these results indicate that in vivo Th1 development can occur in a state of IL-12 unresponsiveness.

Effects of exogenous IL-12 on cardiac allograft rejection. As IL-12 treatment markedly enhanced Th1 responses in WT allograft recipients (Figs. 3 and 4), one might predict that IL-12 treatment would exacerbate allograft rejection. To test this possibility, cardiac allograft function was monitored by daily abdominal palpation in WT or IL-12R$\beta 1^{-/-}$ allograft recipients bearing BALB/c hearts. Cardiac allograft recipients were either left untreated or injected once daily with 1.0 μg of rIL-12. Treatment of WT allograft recipients with this dose of rIL-12 (n=10) resulted in symptoms of cachexia including weight loss (mean decrease=2.0±0.7 g in 1 week), ruffed fur, hunched posture, and decreased activity. In contrast, IL-12R$\beta 1^{-/-}$ allograft recipients exhibited no signs of IL-12-induced toxicity.

The mean cardiac allograft survival in unmodified WT recipients was approximately 8 days (data not shown; 15, 24).
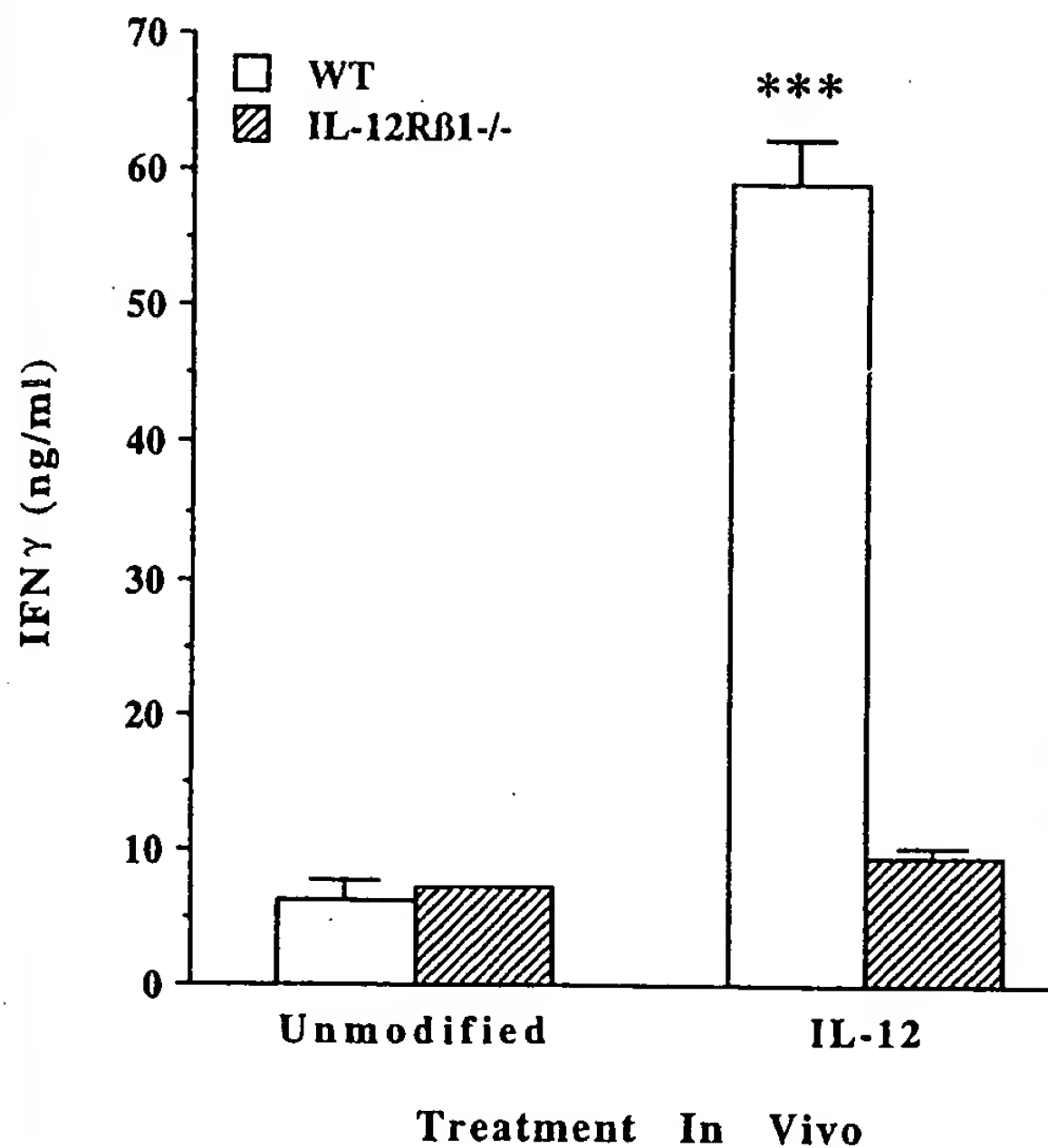
FIGURE 4. Enhancement of in vivo sensitization of IFN-γ-producing cells by IL-12 requires IL-12Rβ1. WT or IL-12Rβ1$^{-/-}$ C57BL/6 cardiac allograft recipients were either left untreated or injected daily with 1.0 μg of rIL-12 on days 1–6 after transplantation. To assess in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from cardiac allograft recipients were restimulated with irradiated BALB/c splenocytes (1×10^6 cells/ml). Supernatants were collected after 72 hr, and the concentration of IFN-γ was determined by ELISA. Results are expressed as the mean concentration of IFN-γ in triplicate samples $\pm$ SD. Data are representative of three separate experiments. ***, $P < 0.005$ (WT unmodified vs. WT IL-12-treated).

Cardiac allografts in IL-12Rβ1$^{-/-}$ recipients were rejected in a similar fashion to that seen in IL-12-deficient mice (15), in that grafts were uniformly rejected by day 7 (n=8). As expected, treatment of IL-12Rβ1$^{-/-}$ allograft recipients with rIL-12 had no effect on the tempo of allograft rejection (n=6). Interestingly, despite the overwhelming Th1 response induced by rIL-12 in WT allograft recipients (Figs. 3 and 4), treatment of these animals with rIL-12 did not appear to accelerate the tempo of graft rejection when compared to grafts of untreated WT recipients on day 7 after transplantation. For example, 7 of 10 (70%) allografts of WT recipients treated with rIL-12 were still functioning on day 7. A histologic evaluation of these grafts revealed similar parameters of early rejection compared to unmodified WT recipients. Specifically, histology was characterized by diffuse mononuclear cell infiltrates, viable myocytes as evidenced by visible nuclei, and relatively uninvolved vessels (Fig. 5). Hence, rIL-12 treatment did not accelerate the pathologic changes associated with acute rejection.

Phenotype of alloantigen-reactive Th1 in WT allograft recipients treated with rIL-12. To determine the phenotype of Th1 responsive to exogenous rIL-12, WT cardiac recipients were depleted in vivo of CD8+ T cells (Fig. 6). Splenocytes obtained from CD8 depleted cardiac allograft recipients produced markedly less IFN-γ upon in vitro restimulation with irradiated donor splenocytes (WT unmodified=6.15 ng/ml vs.
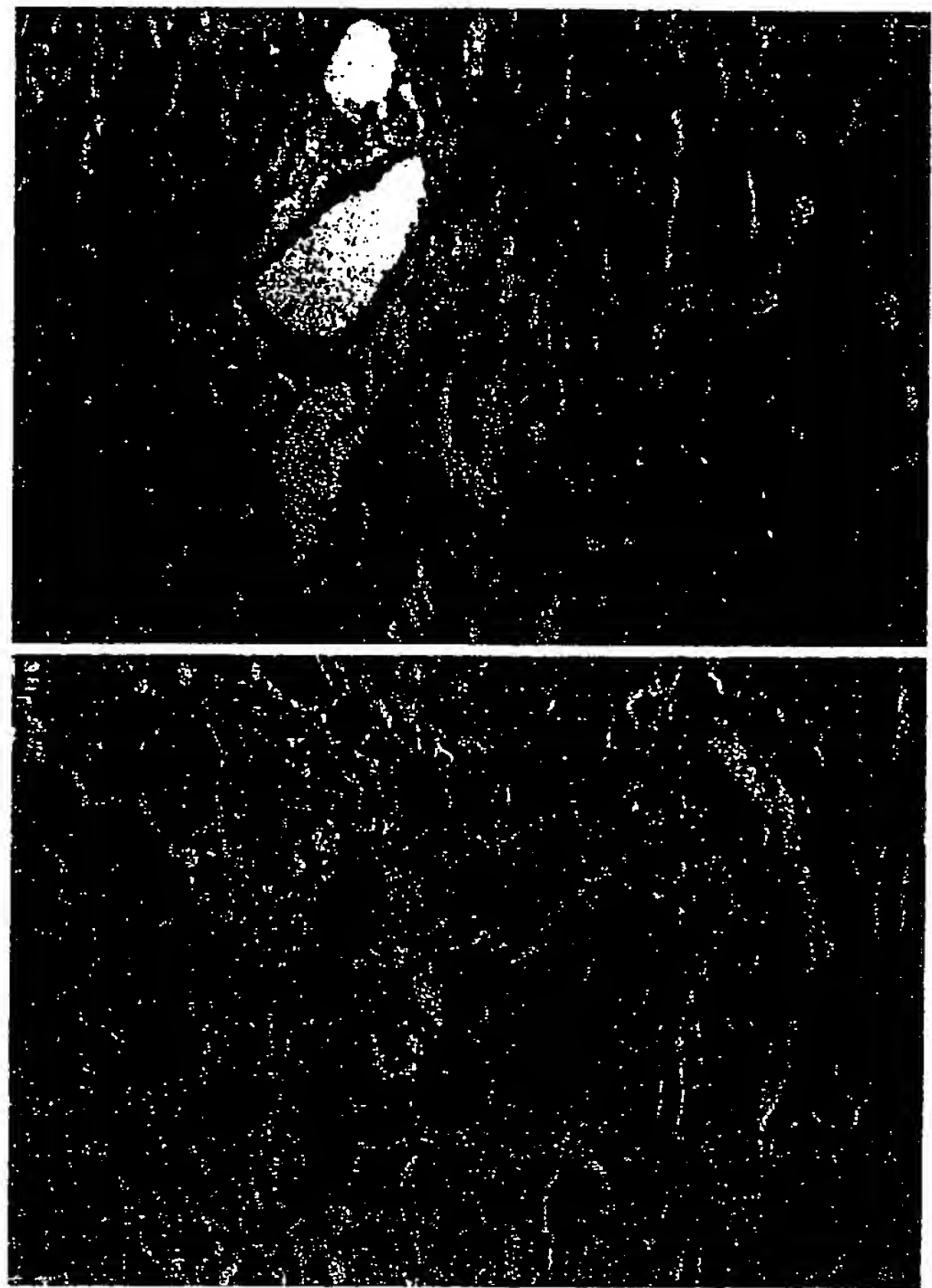
FIGURE 5. Exogenous IL-12 does not exacerbate cardiac allograft rejection. C57BL/6 WT recipients of BALB/c cardiac allografts were either left untreated or injected intraperitoneally with murine rIL-12 (1.0 μg) on days 1–6 after transplantation. On day 7, allografts were harvested for histologic evaluation. (A) H&E-stained section of allografts from WT recipients left untreated (original magnification, ×400). (B) H&E-stained section of allografts from WT recipients treated with rIL-12 (original magnification, ×400). Note in both experimental groups moderate mononuclear cell infiltrates, and relative health of myocytes and vessels. These characteristics are associated with the early phase of acute rejection before onset of myocyte necrosis and vascular damage, which is observed on days 8 or 9 after transplantation. Results are representative of at least 10 individual transplants for each experimental group.

WT anti-CD8 mAb-treated=0.52 ng/ml). Similarly, Th1 that develop as a result of IL-12 stimulation in these experiments were predominantly CD8+ T cells (Fig. 6), as depletion of CD8 cells resulted in a reduction in IFN-γ production (WT IL-12-treated=35.89 ng/ml vs. WT IL-12 plus anti-CD8 mAb-treated=2.75 ng/ml).

IL-12 Treatment (1.0 µg/Day) Inhibits Alloantibody Responses

Given our findings that treatment of WT cardiac allograft recipients with rIL-12 resulted in significant augmentation of serum IFN-γ (Fig. 3) and in vivo priming of Th1 (Fig. 4), one would predict that IL-12 treatment should drive IgG2a alloantibody production. To test this possibility, sera alloantibody production was assessed on day 7 after transplanta-
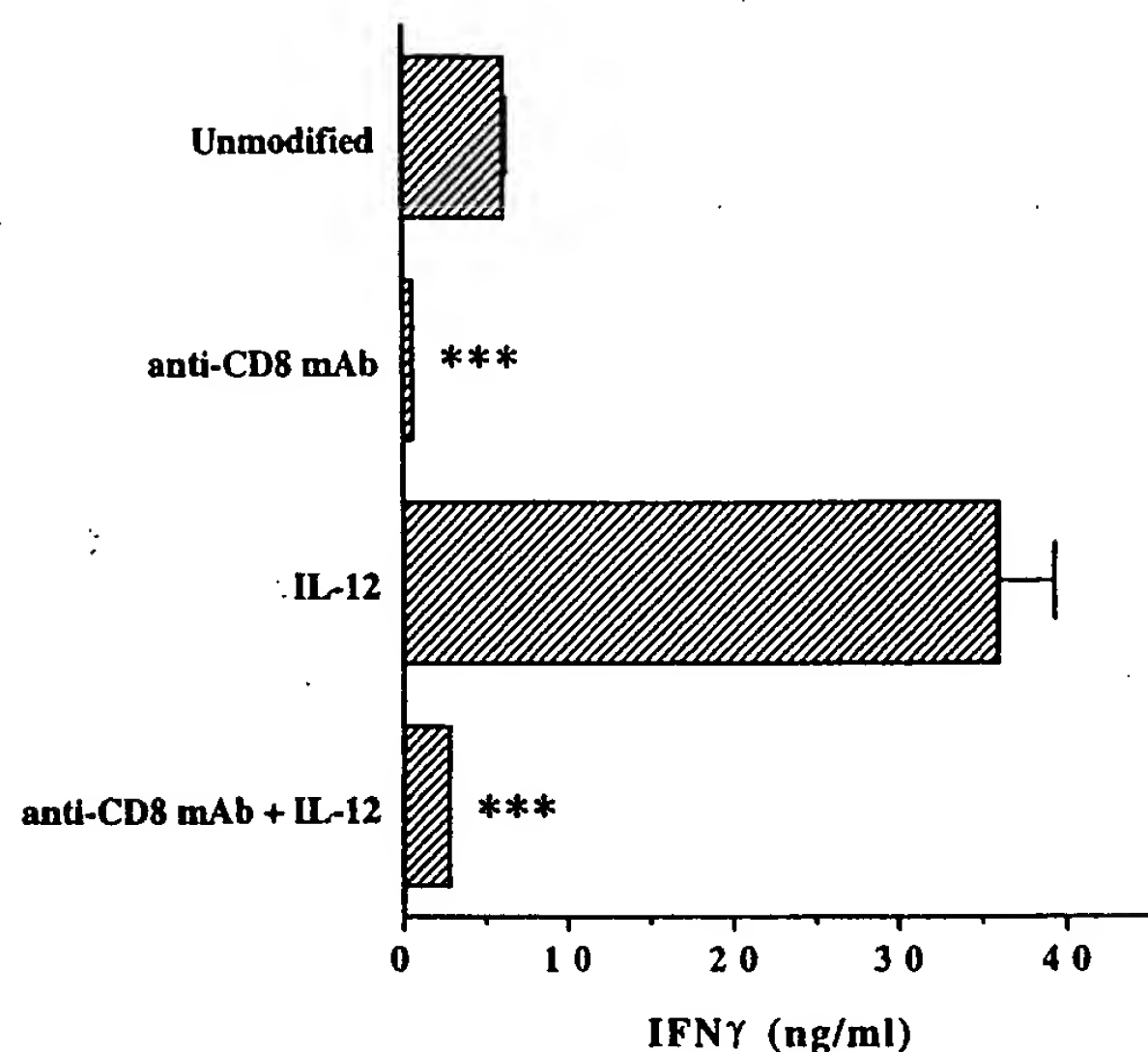
FIGURE 6. Alloantigen-specific Th1 responding to exogenous rIL-12 are CD8+ T cells. In these experiments, splenocytes were obtained from cardiac allograft recipients either left untreated or treated with anti-CD8 mAb, rIL-12, or a combination of anti-CD8 mAb plus rIL-12. Th1 function was assessed by IFN-γ production after a 72-hr restimulation of recipient's splenocytes with irradiated BALB/c splenocytes. Results are expressed as the mean concentration of IFN-γ in triplicate samples ± SD. Data are representative of three separate experiments. ***, P<0.005 (WT unmodified vs. WT anti-CD8 mAb-treated; WT IL-12-treated vs. WT IL-12 plus anti-CD8 mAb-treated).

tion in WT cardiac allograft recipients either left unmodified or treated once daily with 1.0 μg of rIL-12. In these experiments, sera IgG2a was undetectable in rIL-12-treated WT allograft recipients at this time point (data not shown). Further, treatment of WT recipients with rIL-12 resulted in reduced sera IgM alloantibody in three independent experiments, compared to untreated WT recipients (Table 2). This observation indicates that high doses of rIL-12 inhibit, rather than enhance, alloantibody production in this model. In contrast, treatment of IL-12Rβ1$^{-/-}$ allograft recipients with

rIL-12 resulted in a slight increase in sera IgM compared to unmodified knockout recipients.

Effects of Low-dose IL-12 (0.1 μg/Day) on Alloantibody Responses

The reduction in sera IgM in WT allograft recipients after rIL-12 (1.0 μg/day) treatment (Table 2) may have been caused by anti-proliferative or toxic effects on B cell function caused by rIL-12 and/or IFN-γ. To test this possibility, WT cardiac allograft recipients were treated once daily with 0.1 μg of rIL-12 and sera alloantibody levels were assessed on day 7 or 8 after transplantation. No alterations in the pathology of allograft rejection was observed in recipients treated with 0.1 μg of rIL-12 compared to 1.0 μg (data not shown). Further, treatment of WT allograft recipients with 0.1 μg of rIL-12 resulted in significantly less IL-12-induced toxicity, serum IFN-γ concentrations in these animals were undetectable by ELISA, and in vivo Th1 sensitization was similar to untreated allograft recipients (data not shown). These results suggested that the 0.1-μg dose of rIL-12 was ineffective in vivo. However, unlike the higher dose of rIL-12 (1.0 μg), treatment of WT cardiac allograft recipients with 0.1 μg of rIL-12 resulted in an increase in sera IgG2a, but not IgG1 at both days 7 and 8 after transplantation (Table 3). Further, IL-12 treatment augmented sera IgM levels at both time points.

DISCUSSION

Bioactive IL-12 exists as a p70 heterodimer composed of p35 and p40 subunits (28, 29). Each subunit of IL-12 interacts with a distinct component of the IL-12R: p40 binds to IL-12Rβ1 and p35 interacts with IL-12Rβ2 (19, 20). Both receptor subunits are associated with members of the Janus kinase family (30), and therefore may facilitate IL-12-mediated signal transduction. However, most of the attention has been given to IL-12Rβ2, which associates with JAK2 (30). For example, recent evidence supports a requirement for IL-12Rβ2 expression in IL-12-induced phosphorylation of Stat4 (21, 22). Further, these studies revealed the importance of the β2 subunit of IL-12R by demonstrating that the unresponsiveness of Th2 to IL-12 in both human (21) and mouse (22) is a result of loss of IL-12Rβ2 expression by these cells. These results indicate that the binding of the p40 subunit of

TABLE 2. High-dose rIL-12 inhibits IgM production in vivo^a

	Serum IgM (mean channel fluorescence)	
	Unmodified	IL-12 Treatment
WT		
Experiment 1	37.18	9.85
Experiment 2	33.97	11.39
Experiment 3	78.51	27.37
IL-12Rβ1$^{-/-}$		
Experiment 1	17.36	31.52
Experiment 2	33.83	43.64
Experiment 3	21.67	35.96

^{a} Serum was obtained on day 7 after transplantation from WT or IL-12Rβ1$^{-/-}$ cardiac allograft recipients. Animals were either left untreated or injected with 1 μg of rIL-12 once daily. Anti-BALB/c IgM was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence and represent three separate experiments for each group.

TABLE 3. Low-dose rIL-12 augments IgM and IgG2a production in vivo^a

	Mean channel fluorescence		
	IgM	IgG1	IgG2a
Experiment 1 (day 7)			
Unmodified	11.90	2.06	2.58
IL-12	28.94	2.67	12.79
Experiment 2 (day 8)			
Unmodified	31.54	8.53	16.31
IL-12	46.57	9.72	49.00

^{a} Serum was obtained on day 7 or 8 after transplantation from cardiac allograft recipients either left untreated or injected with 0.1 μg of rIL-12 once daily. Isotype-specific anti-BALB/c alloantibody was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence. Mean channel fluorescence for isotype controls were 1.65 (IgM), 1.67 (IgG1), and 1.57 (IgG2a).

IL-12 to IL-12Rβ1 is not sufficient to mediate the bioactivity of heterodimer IL-12. However, we have reported that p40 promotes alloantigen-specific CD8+ Th1 development in the absence of heterodimer IL-12 (*15*). This observation suggests that IL-12 p40 mediates its stimulatory effect through IL-12Rβ1 alone, or that IL-12Rβ1 associates with a yet unidentified component of IL-12R on CD8+ T cells. These possibilities have not been tested. Additional data are emerging that support a biologic role of p40 interacting with IL-12Rβ1 (*1*). Specifically, p35 knockout mice, which are capable of producing p40 in levels similar to WT mice (*31*), are less susceptible to infection with *Listeria* and *Cryptococcus neoformas* compared to p40 knockout mice. Hence, one goal of the current study was to assess the role of IL-12Rβ1 in alloimmune responses both in vitro and in vivo.

IL-12 is a potent stimulator of in vitro alloantigen-specific Th1 development, in that the addition of IL-12 to MLC consisting of WT responder splenocytes resulted in a 10-fold or greater increase in IFN-γ production (Table 1; *15*). Exogenous rIL-12 also markedly augments in vitro Th1 development in mice that are deficient in p35, p40 (*15*) or both subunits of IL-12 (JR Piccotti and DK Bishop, unpublished observations), indicating that T cells of these mice are equipped with a functional IL-12R. In contrast, IL-12 did not alter MLC IFN-γ production by splenocytes of IL-12Rβ1$^{-/-}$ mice (Table 1). This result illustrates the requirement of β1 subunit of IL-12R for IL-12-driven Th1 differentiation in vitro. It should be noted that, although IFN-γ production by IL-12Rβ1$^{-/-}$ splenocytes in MLC was reduced compared to WT values (Table 1), this cytokine was readily detectable by ELISA, suggesting that IL-12 is not an absolute requirement for in vitro Th1 responses.

IL-12 is also a key cytokine involved in promoting cell-mediated immune responses in vivo (*1, 2*). However, what role IL-12 plays in transplant rejection remains unclear. It has been reported that IL-12 has a central role in the progression of acute graft-versus-host disease (GVHD) in mice (*6, 32*). In these studies, neutralizing IL-12 with a polyclonal anti-IL-12 antibody results in the amelioration of acute GVHD (*32*) and, conversely, treatment with exogenous IL-12 converts chronic GVHD into exacerbated acute GVHD (*6, 32*). Further, Williamson et al. (*33*) have reported that neutralizing IL-12 during the inductive phase of GVHD results in a Th1 to Th2 shift evidenced by a reduction in IFN-γ and enhancement of IL-5 and IL-10 production by Con A-stimulated splenocytes. In contrast to these findings, neutralizing IL-12 in mouse vascularized cardiac allograft recipients promotes intragraft Th2 cytokine (IL-4 and IL-10) gene expression; however, these grafts are rejected in an accelerated fashion compared to untreated recipients (*16*). Importantly, in vivo Th1 priming is not inhibited by IL-12 neutralization, indicating that Th1 development can occur independent of IL-12 (*16*). This possibility is further supported by the observation that splenocytes of IL-12Rβ1$^{-/-}$ allograft recipients produce similar concentrations of IFN-γ upon restimulation with donor splenocytes compared to WT recipients (Fig. 4). It does not appear that Th1 development in IL-12Rβ1$^{-/-}$ mice is a result of the interaction of endogenous IL-12 with the low-affinity IL-12Rβ2, as treatment of these animals with rIL-12 did not augment in vivo priming of IFN-γ-producing cells (Figs. 3 and 4).

A second hypothesis tested in the current study was treatment of cardiac allograft recipients with IL-12 would accelerate the rejection process as a result of exacerbated Th1-driven immune responses. Administration of exogenous rIL-12 significantly augmented in vivo sensitization of IFN-γ-producing cells in WT cardiac allograft recipients, as evidenced by increased sera IFN-γ (Fig. 3) and enhanced production of IFN-γ by splenocytes after restimulation with donor alloantigens in vitro (Fig. 4). However, this fulminate Th1 response in vivo did not result in anticipated acceleration of graft rejection when compared to untreated control recipients (Fig. 5). It is possible that induction of high systemic levels of IFN-γ results in an inhibition of immune response as a result of IFN-γ's anti-proliferative properties on effector cell development (*34*). However, graft survival was not prolonged after rIL-12 treatment in the current study. This observation questions the overall importance of Th1 responses in this experimental model, and suggests that the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection. Indeed, Th2-driven immune responses are emerging as potential effector cells of rejection in both human and experimental transplantation (reviewed in *35*).

Finally, we examined the influence of rIL-12 administration on allospecific B cell function. In an experimental system in which PVG.RT1^u congenic rats were immunized with an isolated alloantigen, Gracie et al. (*36*) reported that treatment with murine rIL-12 (1.0 μg/day for 5 days) after allo-immunization augments levels of allospecific IgG2b and IgG2c, while decreasing IgG1. The authors demonstrated that co-administration of neutralizing anti-IFN-γ mAb abrogated this response, indicating that the enhancement of B cell function by IL-12 was dependent on IFN-γ. When adjusted for body weight, this dose of rIL-12 in the rat is comparable to our 0.1-μg dose in the mouse. In the current study, treatment of WT cardiac allograft recipients with 0.1 μg of rIL-12/day for 6 days increased the level of sera IgG2a compared to untreated recipients on day 7 and 8 after transplantation (Table 3). However, administration of 1.0 μg of rIL-12/day reduced allospecific B cell function, indicated by a decrease in sera IgM (Table 2) and absence of isotype switch to IgG2a. These observations suggest a biphasic response to IL-12 treatment in WT mouse cardiac allograft recipients likely dependent on the concentration of IFN-γ.

In summary, this study illustrates that the β1 subunit of mouse IL-12R is critical for IL-12-driven alloimmune responses both in vitro and in vivo, and that IL-12Rβ2 alone does not transduce IL-12 signaling. These observations are supported by recent reports, which have shown that humans deficient in IL-12Rβ1 exhibit severe impairment in their resistance to infections as a result of intracellular pathogens (*37, 38*). The generation of mice deficient in IL-12Rβ2 will provide an important animal model to evaluate whether β1 subunit of IL-12R alone conveys IL-12 responsiveness in vivo. Specifically, these mice would be useful in determining the mechanism by which p40 subunit of IL-12 enhances CD8+ Th1 development (*15, 16*). Finally, this study questions the importance of Th1-driven alloimmune responses in cardiac allograft rejection, as exacerbated Th1 responses induced by IL-12 failed to accelerate graft rejection in this model.

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Zinc Inhibits the Mixed Lymphocyte Culture

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ABSTRACT

The mixed lymphocyte culture (MLC) is an established clinical method for bone marrow transplantation, as it serves as an in vitro model for allogenic reaction and transplantation. We previously showed that cytokine release into the supernatant is a more specific and sensitive parameter for cross-reactivity in the MLC than the common measurement of cell proliferation. Therefore we tried to find an inhibitor of the MLC in vitro with the least side effects in vivo, measuring interferon (IFN)-γ as one of the most important cytokines in posttransplant medicine. Earlier studies showed that zinc is an important trace element for immune function with both stimulatory and inhibitory effects on immune cells. We found that slightly elevated zinc concentrations (three to four times the physiological level), which do not decrease T-cell proliferation in vitro nor produce immunosuppressive effects in vivo, suppress alloreactivity in the mixed lymphocyte culture. In this report we analyzed the mechanism whereby zinc influences the MLC to possibly find a nontoxic way of immunosuppression.

Index Entries: Mixed lymphocyte culture (MLC); mixed lymphocyte reaction (MLR); trace elements; zinc.

INTRODUCTION

The mixed lymphocyte culture (MLC) is a well-established and important tool for determination of compatibility between host and donor

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in transplantation medicine, as it serves as an in vitro model for allogenic reaction (1,2). It is common to measure T-cell proliferation in the MLC, but it was recently shown that cytokines are more specific and sensitive parameters for the prediction of a possible graft rejection, as they play a critical role in the posttransplant response (3–5). The TH1-cytokine interferon-γ (IFN-γ) was identified as the most important factor within the cytokine cascade in the MLC (6). It is known to induce cytotoxic T-lymphocytes (CTL) (7) by enhancing the expression of both major histocompatibility complex (MHC) class I and MHC class II molecules (8). The IFN-γ response mainly depends on HLA-DR differences and it therefore well represents reactivity between two individuals in the MLC (9).

In transplantation medicine, cyclosporin A, FK506, and other substances are used to prevent graft rejection. In vitro experiments revealed an inhibition of the MLC (10), but, unfortunately, all of these immunosuppressants show a wide range of toxicities in vivo, such as nephrotoxicity, neurotoxicity, and, probably, carcinogenicity (11–13). As we are beginning to understand the molecular mechanisms of cyclosporin A and FK506 function better and better, one of the major aims is to find similar substances with less toxicity.

Zinc within the physiological range (12–16 μM) is an important trace element for immune function (14). Zinc deficiency in vivo could be linked to various clinical symptoms such as impaired immune response with regard to decrease in number, differentiation, and function of T-lymphocytes and natural killer (NK) cells as well as decreased activation of monocytes and phagocytosis by macrophages, resulting in a high incidence of bacterial, viral and fungal infections. These symptoms, in the most severe form shown in the hereditary disease acrodermatitis enteropathica caused by malabsorption of zinc, are completely reversible after adequate substitution of zinc (15). On the other hand, high concentrations of zinc (about eight times the physiological level) led to cytotoxic effects with impairment of all T-cell functions, and inhibition of monokine induction by superantigens such as zinc is also important for the binding of some bacterial superantigens to the β-chain of the MHC class II molecule (16,17). Optimal immune-cell function hence requires a well-balanced zinc level.

In the following study, we investigated whether zinc is able to impair alloreactivity in the MLC at concentrations with neither cytotoxic effects in vitro nor toxic side effects in vivo.

MATERIALS AND METHODS

Preparation of Lymphocyte Cultures

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density centrifugation over Ficoll–Hypaque (Biochrom, Berlin, Germany), washed twice with phosphate-buffered saline (PBS, Gibco, Berlin, Germany) and resuspended in RPMI-1640 medium (Biochrom) supplemented with 10% heat-inactivated fetal calf

serum (FCS, low endotoxin, myoclone quality; Life Technologies, Eggenstein, Germany), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all obtained from Biochrom, Berlin, Germany). The cells were adjusted to a final concentration of 2×10^6 cells/mL. Equal volumes of cell suspensions of two donors were seeded in samples to a final volume of 1 mL into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). For controls, 1 mL of the adjusted cell suspension was cultured separately. The cultures were incubated for 5 d at 37°C in a 5% humidified CO_2 atmosphere after addition of the appropriate amount of zinc.

Zinc Preparations

Zinc sulfate (Sigma, Deisenhofen, Germany) was dissolved in sterile water to achieve a zinc stock solution of 10 mM. This solution was further diluted in unsupplemented protein-free medium (PFM, Ultradoma, BioWhittaker) at a ratio of 1 to 2 and then sterile filtered. To achieve the final concentrations, PFM was used. The zinc solution was added to the cultures in a volume of 10% of the final culture volume.

Determination of Cytokines

The culture supernatants were harvested after 5 d and stored at –80°C. The quantification of the cytokine release into the supernatant was performed by enzyme-linked immunosorbent assay (ELISA) technique (for IFN-γ provided by Bender Med Systems, Vienna, Austria). Results were measured in picograms per milliliter at 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Flow Cytometry

Propidium iodide (PI) staining was performed by using a stock solution of 1 mg/mL (PI, Sigma). Cells (1×10^6/mL) were incubated with 10 µL of PI stock solution for 20–30 min to allow intercalation of PI in double-stranded DNA. Finally, PI staining was measured at a wavelength of 620 nm in a flow cytometer (Coulter, Krefeld, Germany).

Statistical Analysis

The results are expressed as median values. The significance is taken by Student's t-test analysis.

RESULTS

Influence of Zinc on Mixed Lymphocyte Cultures

We harvested the supernatants of zinc-supplemented mixed lymphocyte cultures (MLC) on d 5, proven to be the maximum of the IFN-γ secretion *(18)*. Analyzing IFN-γ release in 20 MLC experiments supplemented with different concentrations of zinc, we found expected amounts of IFN-
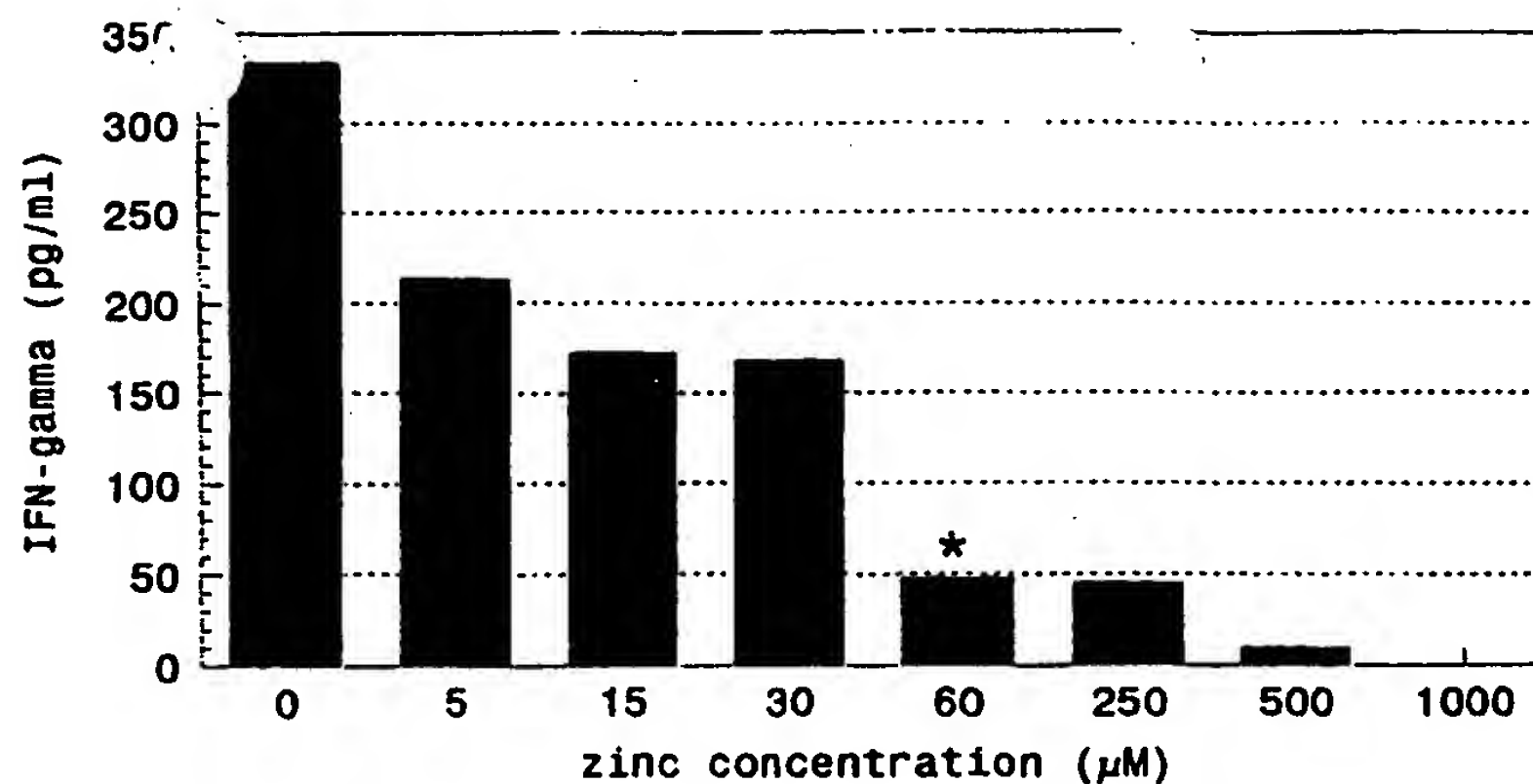
Fig. 1. IFN-γ secretion in the MLC after zinc supplementation. Zinc concentrations up to 1 mM were added to mixed lymphocyte cultures; controls remained unsupplemented. Secretion of IFN-γ in the culture supernatants was determined by ELISA after 5 d of culture. Median values of n = 20 experiments are expressed in picograms per milliliter. Significance was calculated by the Student's t-test (*p = 0.0017).

γ (334 pg/mL) in the supernatant of control MLC without zinc addition, whereas increasing zinc concentrations led to a dose-dependent reduction of the IFN-γ level. At 60 μM, the IFN-γ production was significantly diminished (48 pg/mL, p = 0.0017); at 500 μM, no IFN-γ was detectable (Fig. 1).

In order to prove our hypothesis that this result—that zinc concentrations of 60 μM inhibit the MLC—was the result of a specific effect in the MLC and not to a loss of T-cell vitality, we added zinc concentrations of up to 5 mM to PBMC and measured cell viability by flow cytometry after an incubation time of 48 h. Figure 2 shows that 93.2% of the cells are still vital after addition of 50 μM zinc and 92.3% with medium supplementation of 100 μM zinc compared to controls without zinc addition with 91.3% viability. Zinc concentrations as high as 250 μM causes a reduction of cell survival of 33% (Fig. 2).

For further analysis of possible mechanisms responsible for this inhibition, we preincubated PBMC with 50 μM zinc for 20 min and then co-cultured these two populations in the MLC. The results reveal a marked influence of the point of time at which zinc is added to the culture: Preincubation of PBMC led to a greater reduction of IFN-γ than simultaneous zinc supplementation to the MLC (Fig. 3).

DISCUSSION

The human mixed lymphocyte culture (MLC) is an important method to test donor–recipient compatibilty in bone marrow transplantation. It could be shown that cytokine release, especially IFN-γ, has a very good predictive value with regard to the transplantation outcome (3), as cytokines play a major role in the generation of an alloreactive immune response and
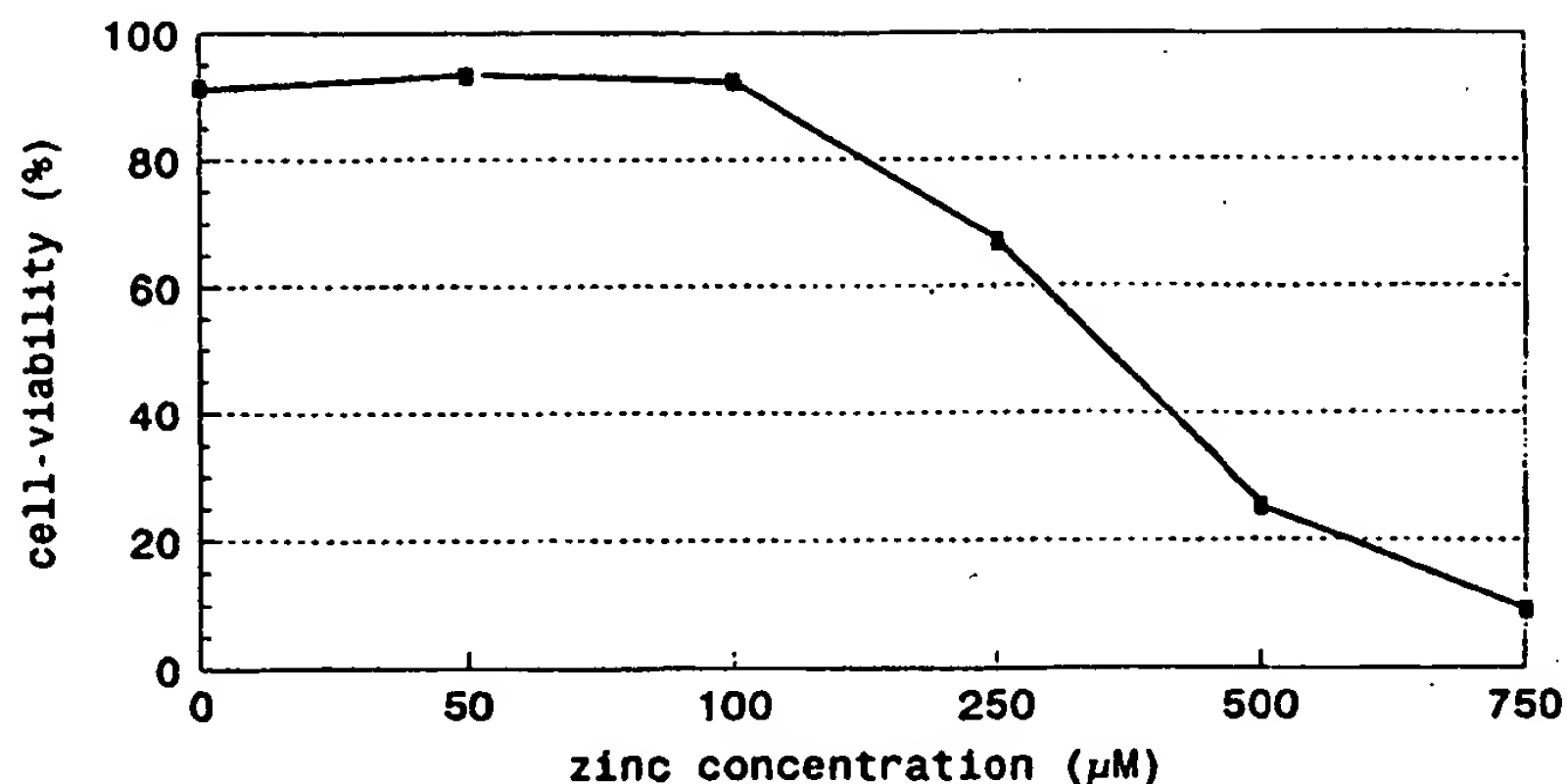
Fig. 2. Viability of PBMC after zinc supplementation. Zinc concentrations of up to 750 µM were added to unstimulated PBMC; controls remained unsupplemented. Cell viability was determined by flow cytometry after an incubation time of 48 h. One representative experiment is shown, values are expressed in percent of the total cell population.
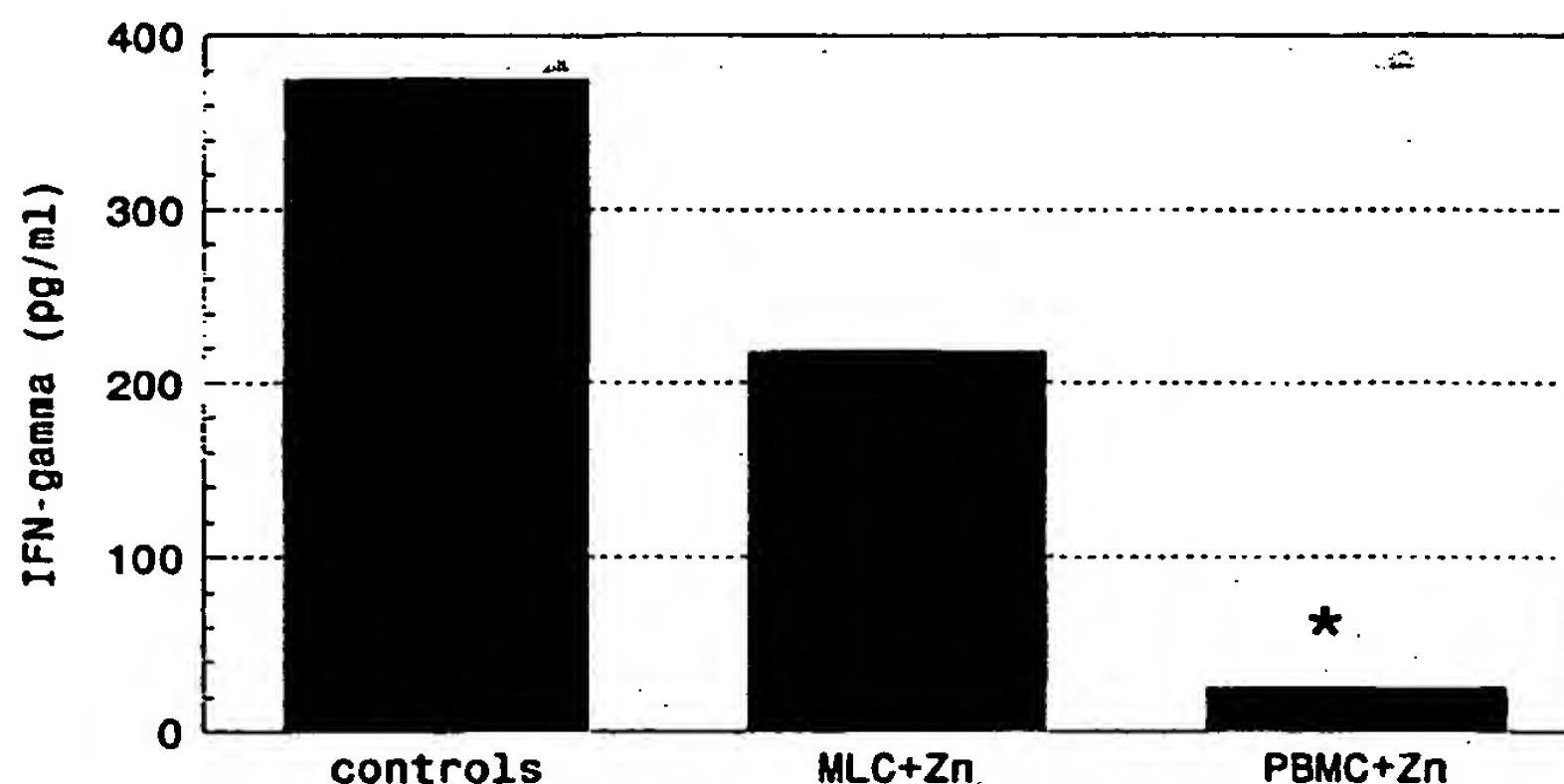

Fig. 3. Effect of preincubation of PBMC with zinc. Zinc in a concentration of 50 µM was added to the MLC simultaneously (MLC + Zn); PBMC was preincubated with 50 µM zinc for 20 min and then cocultured in a MLC (PBMC + Zn); controls remained unsupplemented. Medians of n = 10 experiments are presented. Significance was calculated by the Student's t-test (*p = 0.01).

for the induction of graft rejection in vivo (4,5). Taking this in vitro model, it has always been the aim to inhibit proliferation of immune cells in order to find a way to prevent graft rejection in transplantation medicine. Landolfo et al. inhibited T-cell reactivity by the addition of anti-IFN-γ both in vitro and in vivo (19); others showed a reduced graft rejection rate by repeated injections of monoclonal anti-IFN-γ antibodies in a skin-, heart-, or pancreas-tissue transplantation situation (20–22).

In vivo substances like cyclosporin A or FK506 are broadly applied, as they are capable of prolonging graft survival. In vitro, they show an inhibitory effect on T-cell proliferation in the MLC (10). Yet, all of these therapeutical agents cause major side effects (e.g., nephrotoxicity, neurotoxicity, and others), which lead to a limitation of their use (11–13).

Zinc is an essential trace element with great influence on immune function. The physiological plasma level of zinc ranges from 12 to16 µM. In our study, we applied zinc concentrations up to 100 µM, which can be reached by pharmacological application of zinc in vivo without causing side effects (23).

We found that zinc concentrations of 60 µM, four times the physiological level, inhibit alloreactivity in the MLC. It is unlikely that the reduction of IFN-γ is the result of a loss of T-cell activity, as it could be shown earlier that T-cells are still able to proliferate in medium supplemented with zinc concentrations as high as 100 µM (24). Furthermore, we analyzed the viability of the PBMC by flow cytometry, showing that a concentration of 250 µM is required to reduce cell viability by 33% (Fig. 2).

Increased zinc levels of over 100 µM cause unstimulated human PBMC to release cytokines (25). This stimulatory effect of zinc is only seen in the presence of accessory cells, especially monocytes, as mostly IL-1 proved to be an essential cosignal for T-cell activation by zinc. Higher concentrations of zinc impair all T-cell and monocyte function by inhibition of the IL-1 receptor type I-associated protein kinase (IRAK), thus blocking the intracellular signal transduction pathway at a very early stage (24).

In our study, we applied zinc in concentrations that neither show cytotoxic effects nor reach stimulatory level. Therefore, there seems to be a specific effect of zinc on the responding T-cells in the MLC.

The results of earlier studies proposed an oligoclonal pattern of T-cell stimulation in the MLC similar to T-cell activation by superantigens (3). Furthermore, a highly altered Vβ repertoire of T-cells infiltrating long-term rejected kidney allografts were discribed (26). Superantigens bind directly and partially with high affinity to major histocompatibility complex (MHC)-class II proteins, especially to HLA-DR. T-Cell activation is achieved by the formation of a complex of the Vβ-chain of the T-cell receptor (TCR), the MHC molecule, and the superantigen. This binding is regulated by zinc, as zinc itself does not interact with the MHC molecule directly (27). We previously showed that the HLA-DR and HLA-DQ-molecules have the greatest influence on cytokine release in the MLC and thus on the outcome of a transplantation in vivo (9).

There are two main possible explanations for the phenomenon described. First, zinc in the applied concentration could saturate the MHC and, therefore, prevent a binding between TCR and MHC. In order to prove this hypothesis, we preincubated PBMC with zinc and then cocultured these populations. If an extracellular mechanism were actually responsible for the inhibition of the MLC, we would expect no significant difference in IFN-γ secretion in either setting. Preincubation of PBMC

resulted in a markedly lower IFN-γ secretion than the culture of two PBMC populations with simultaneous zinc supplementation to the MLC (Fig. 3), so that it seems more likely that zinc interferes with the intracellular signal transduction in the MLC. Therefore, zinc may regulate the alloreactivity of T-cells and might be an explanation for increased preterm delivery and abortion in zinc-deficient pregnant women *(28,29)*. As mentioned earlier, higher concentrations of zinc are able to block the intracellular signal transduction pathway by inhibition of IRAK. We propose that the stimulation of T-cells by an HLA-different cell population can be blocked by zinc via specific inhibition of phosphorylation processes, leading to a diminished signal transduction in the cell. This results, among other things, in reduced secretion of cytokines, which should lead to less graft rejection in vivo. Various protein kinases such as cAMP- and cGMP-dependent protein kinases as well as protein tyrosine kinases are involved in zinc-induced cell stimulation and zinc also influences gene expression of different immunologically relevant transcription factors such as nuclear factor (NF)-κB and metallothionein transcription factor (MTF-1) as well as others. Which alteration of signal transduction zinc exactly inhibits the MLC remains the subject of further investigation. Because the MLC is inhibited by very low zinc concentrations, this inhibitory effect seems to be a specific pathway.

In conclusion, zinc could become an immunosuppressant in transplantation medicine without toxic side effects, which still leaves the immune system with the ability for phagocytosis. The infection rate will therefore be reduced compared to current immunosuppression. However, this has yet to be proven in in vivo transplantation models.

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Basic & Clinical
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different PTKs. Phosphorylation is a common mechanism of regulating protein function; indeed, many of the PTKs must themselves be phosphorylated before they can become active. Several important types of lymphocyte surface receptors (including membrane immunoglobulin and T cell receptor proteins) are physically linked to specific cytoplasmic PTK proteins, which become active when the receptor binds its target ligand. These receptor-associated PTKs, in turn, may then activate other types of PTKs through phosphorylation, so that almost immediately a host of different PTKs are recruited into the response. By phosphorylating still other types of substrates, such as proteins that control cytoskeletal organization, expression of specific genes, and entry into the cell cycle, these newly activated PTKs appear to be either directly or indirectly responsible for triggering all subsequent events in lymphocyte activation. At present, however, the functions of most individual PTKs are uncertain.

One almost immediate effect of the PTK cascade is to activate the enzymatic breakdown of a specific class of phospholipids (called phosphatidylinositides) within the cell. The products of this hydrolysis include 2 small organic molecules, **diacylglycerol (DAG)** and **inositol 1,4,5-trisphosphate (IP$_3$)**, which are released into the cytoplasm and serve as second messengers to trigger additional changes in cellular physiology. Among the most notable changes is a rapid, marked increase in the concentration of **intracellular free calcium ions,** which flood into the cytosol from organellar storage pools and from the extracellular medium, reaching maximal concentrations within 1 minute after contact with the activating stimulus. Like PTK activation, these rapid calcium fluxes are thought to be critical for initiating the subsequent events in activation.

Within the first hour after stimulation, the rates of oxidative metabolism and of overall protein and RNA synthesis in the lymphocyte rise. The chromatin begins to decondense as previously silent genes are transcribed and the cell prepares to undergo mitosis. After 2–4 hours, specific proteins that are thought to regulate cell proliferation, such as the product of the proto-oncogene c-*myc,* become detectable in the nucleus. In parallel with these biochemical events, the morphology of the cell changes in a process known as **blast transformation**: its overall diameter increases to 15–30 μm as both its nucleus and cytoplasmic enlarge; the nuclear chromatin becomes loose and pale-staining; and the cell acquires a prominent nucleolus (reflecting a high rate of RNA synthesis). Within 8–12 hours, the changes are sufficiently marked that the cell can be recognized under the light microscope as a **lymphoblast**—a lymphocyte poised to begin mitosis. DNA synthesis takes place at around 18–24 hours after stimulation. The first cell division occurs 2–4 hours later and, depending on the conditions, can be repeated 5 or more times in succession, at intervals as

brief as 6 hours. The effector cells produced as a result of each division mature completely within a few days and express the immune functions typical of their lineage for several days thereafter.
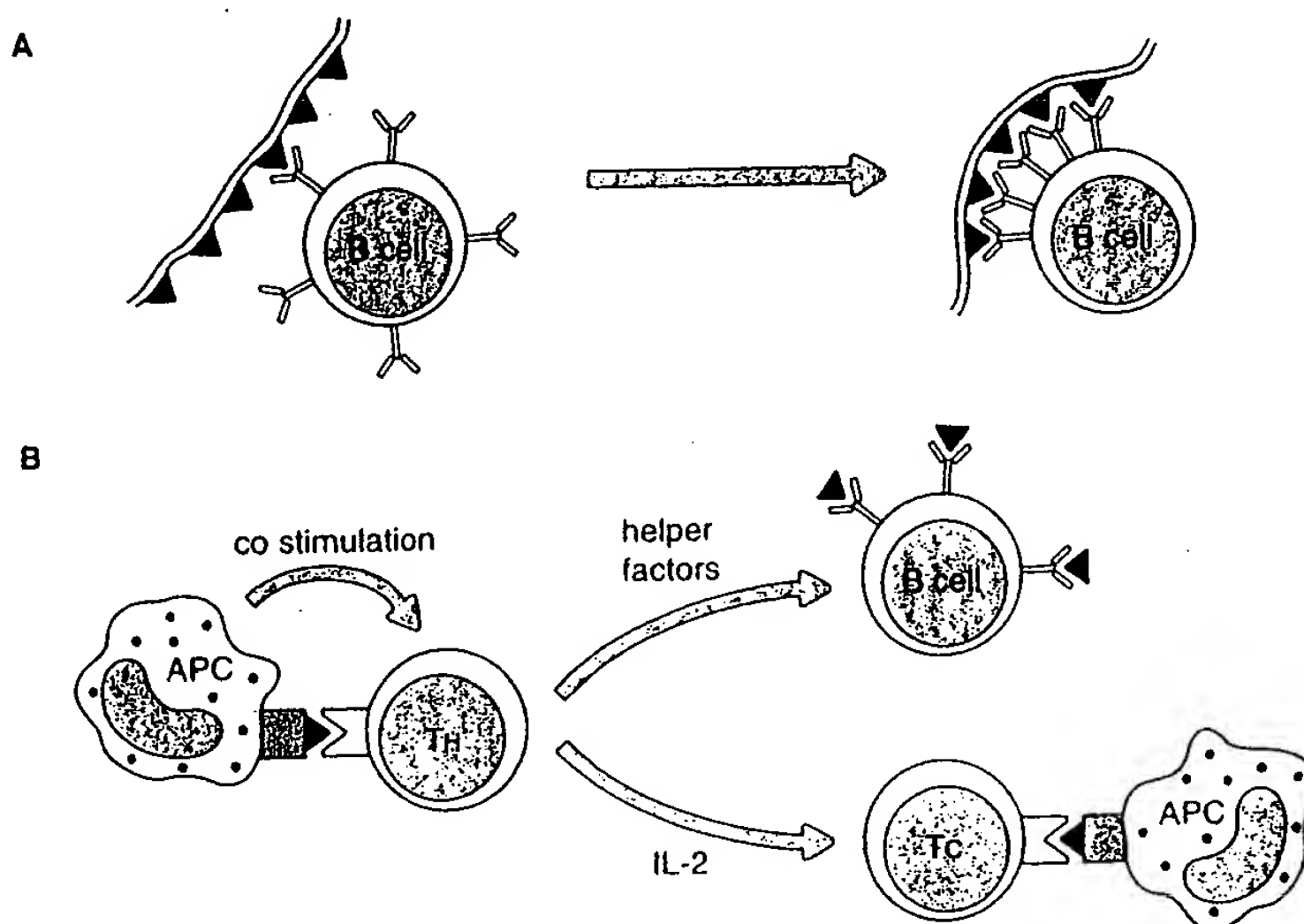
REQUIREMENTS FOR ACTIVATION OF B OR T LYMPHOCYTES

What are the stimuli that can lead to lymphocyte activation in vivo? Certainly, the most important are the innumerable foreign **antigens** that are recognized and bound by membrane immunoglobulins or T cell receptor proteins. A few types of antigens are in themselves sufficient to activate B cells—these are usually highly polymeric proteins or polysaccharides that are able to interact simultaneously with many immunoglobulin proteins on the surface of a single cell. Such multivalent antigens act to **cross-link** the immunoglobulins to one another, so that eventually a great many immunoglobulins are gathered at one pole of the cell surface at the point of contact with antigen—a phenomenon known as **capping** (Fig 2–9A). This dense local aggregation of immunoglobulins, each of which is bound to antigen, transmits a very effective signal and is enough to trigger B cell activation.

Activation can also be induced under artificial conditions by cross-linking other types of surface molecules (Table 2–4). Among the agents used for this purpose are certain lectins (sometimes called **mitogens**), which can activate T and/or B cells by cross-linking surface glycoproteins. Similar results can be obtained by using multivalent antibody complexes to cross-link some T cell surface proteins (such as CD3) that are able to transmit signals to the cytoplasm. Alternatively, lymphocytes can be activated pharmacologically by being treated with agents that directly in-

Table 2–4. Mitogens and other conditions used to activate lymphocytes in vitro.

Mitogen or Condition	Specificity
Lectins	
Concanavalin A	T cells
Helix pomatia lectin	T cells
Phytohemagglutinin	T cells; few B cells
Pokeweed mitogen	T and B cells
Wheat germ agglutinin	T cells
Artificial cross-linking of specific surface proteins	
Immunoglobulins	B cells
T cell surface markers (eg, CD3)	T cells
Pharmacologic agents	
Phorbol myristyl acetate plus calcium ionophore (eg, ionomycin)	T and B cells

Figure 2–9. General requirements for lymphocyte activation. *A:* Some highly polymeric antigens that cross-link multiple antigen receptors are sufficient to activate B cells. *B:* Activation by a monomeric antigen requires additional stimuli supplied by another cell type. Costimulators from the antigen-presenting cell (APC) are necessary to activate a TH cell, which in turn provides helper factors for B cells and/or interleukin-2 (IL-2) for Tc cells.

duce calcium fluxes and other important signaling events, thereby bypassing the surface receptors entirely. Such potent artificial activators are often used in clinical testing to study lymphocyte responses in vitro.

The majority of antigens encountered in nature, however, are not polymeric and so do not cross-link receptors. Even when many copies of such an antigen bind individual immunoglobulins on a B cell, they generate only an incomplete signal, which fails to activate the cell. B cells can be activated by these more common antigens only if they are simultaneously stimulated by a nearby activated helper T lymphocyte. This stimulation may be delivered by lymphokines secreted from the T cell, but is transmitted most efficiently through direct contact of the B cell with T cell surface proteins. In either case, the helper-cell-derived proteins (which will be referred to in this book as **helper factors**) interact with non-immunoglobulin receptors on the B cell to generate a second signal. The combined effects of the helper factors and the bound antigen then act synergistically to cause B cell activation.

In a similar manner, T lymphocyte responses to most antigens also require 2 types of stimuli simultaneously. The first is provided by the antigen, which, if appropriately displayed by MHC proteins on an antigen-presenting cell, can be recognized and bound by T cell receptors. When it binds an antigen-MHC complex, the T cell receptor sends a signal to the cell interior, but this signal alone is usually not enough to

cause activation. For helper T cells, full activation also requires contact with other specific ligands, known as **costimulators**, that are expressed on the surface of the antigen-presenting cell. Activation of a cytotoxic T cell, on the other hand, generally requires **IL-2**, a cytokine secreted by activated helper T cells.

In summary, it is important to recognize that activation of a lymphocyte is controlled not only by antigen binding but also by interactions with other cells (Fig 2–9B): all T cells must cooperate with antigen-presenting cells, whereas B cells and cytotoxic T cells depend on helper T lymphocytes. These interactions either require direct surface-to-surface contact or are mediated by highly labile cytokines that act only over extremely short distances. Owing to this interdependence among cell types, lymphocyte activation occurs most commonly and efficiently in the secondary lymphoid organs, where lymphocytes, antigens, and antigen-presenting cells encounter one another at close quarters.

LYMPHOID ORGANS

Lymphocytes are normally present in the blood at a concentration of approximately 2500 cells/mm^3 and so account for roughly one-third of all peripheral white blood cells. Each individual lymphocyte, however, spends most of its life within solid tissues, entering the circulation only periodically to migrate from one resting place to another. Indeed, at any
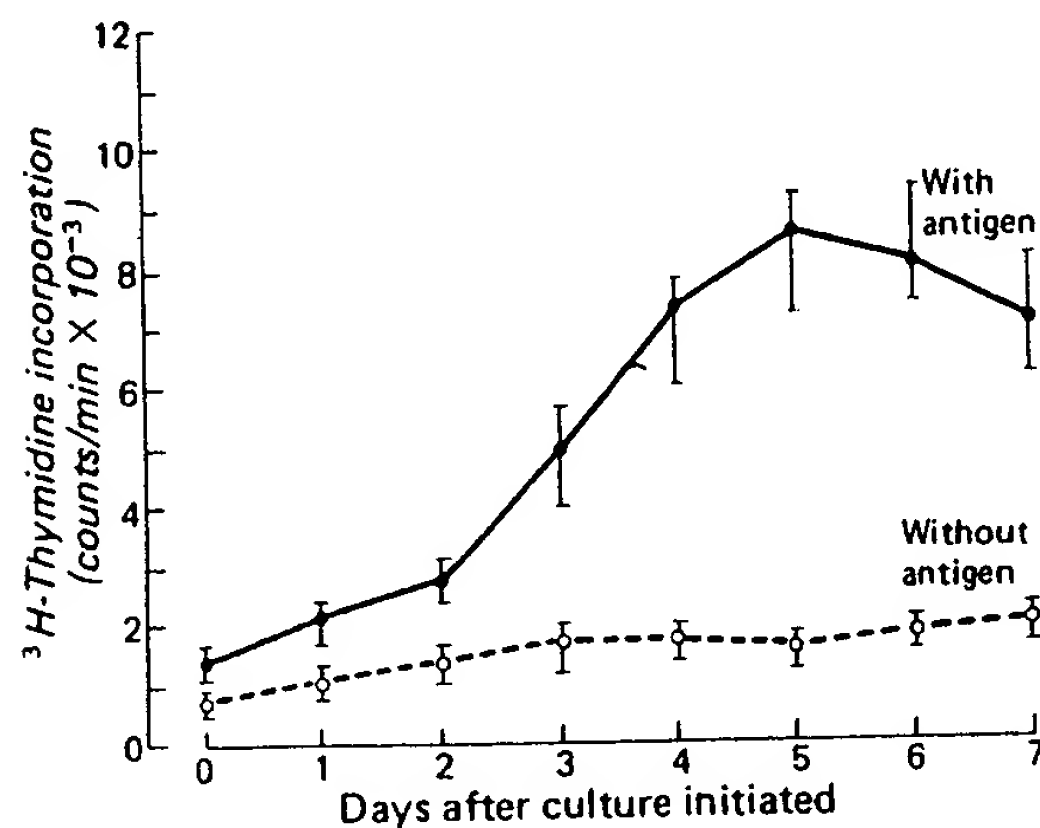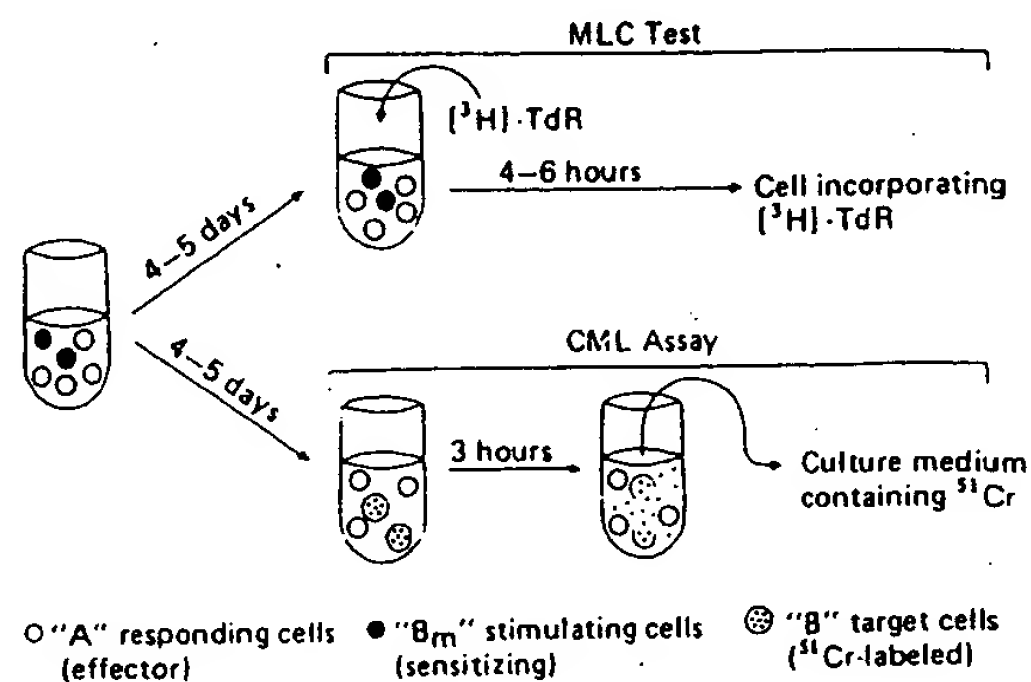
Figure 13-8. Time-response curve for antigen stimulation of 10^6 lymphocytes. Responses of peripheral blood lymphocytes from 15 normal adults with delayed hypersensitivity to the antigen. Cells were cultured as described in the legend to Fig 13-7. Antigen concentration for all cultures was 15 μg/mL. Maximal response occurred on days 5-7 of culture. Results are plotted as the mean ± 1 SD. for 15 individual determinations.

Figure 13-9. MLC and CML assays schematically represented. Cells (black and white balls) from separate individuals are cultured. In MLC, DNA synthesis in responding (noninactivated) cell is measured. In CML assay, the ability of "A" cells to kill ^{51}Cr-labeled "B" cells is measured. See the text for further explanation. [^3H]-TdR, tritiated thymidine. (Reproduced, with permission, from Bach FH, Van Rood JJ: The major histocompatibility complex: Genetics and biology. N Engl J Med 1976;295:806, 872).

until the culture has been allowed to continue for 5-7 days. Fig 13-8 clearly illustrates both the usefulness of and the necessity for performing careful time- and dose-response kinetics in assessing human lymphocyte function.

MIXED LYMPHOCYTE CULTURE & CELL-MEDIATED LYMPHOLYSIS

Mixed lymphocyte culture (MLC) is a special case of antigen stimulation in which T lymphocytes respond to foreign histocompatibility antigen on unrelated lymphocytes or monocytes. This test is performed as either a "one-way" or "2-way" assay (Fig 13-9). In the one-way MLC, the stimulating cells are treated with either irradiation (~ 2000 R) or mitomycin to prevent DNA synthesis without killing the cell. The magnitude of the response is then entirely the result of DNA synthesis in the nonirradiated or non-mitomycin-treated cells. In the 2-way MLC, cells from both individuals are mutually stimulating and responding, DNA synthesis represents the net response of both sets of cells, and the individual contributions cannot be discerned. The culture conditions, time of exposure, ^3H-Tdr pulse labeling, and harvesting procedures are usually identical to those for antigen stimulation. Controls include coculture of syngeneic irradiated and nonirradiated pairs and coculture of allogeneic irradiated pairs. The first control provides baseline DNA synthesis, and the second ensures ade-

quate inactivation by irradiation (or mitomycin) of the stimulator cells.

In the use of MLC as a test for T cell function, difficulties in quantitation often arise owing to variations in stimulator cell antigens that determine the degree of genetic disparity between stimulator and responder cells. To overcome this difficulty and produce a more standardized test, frozen aliquots of viable pooled human allogeneic cells have been employed as stimulator cells.

The stimulating antigens on human cells are class I MHC molecules encoded by the HLA-D locus (see Chapter 5). Responding cells are primarily T lymphocytes with obligate macrophage cooperation. B cells can also respond in MLC, since a marked increase in immunoglobulin synthesis can be detected. MLC may be used as a histocompatibility assay (see Chapter 16) and as a test for immunocompetence of T cells, particularly in immunodeficiency disorders (see Chapters 20 and 21).

Cell-mediated lympholysis (CML) is an extension of the MLC technique in which cytotoxic effector cells generated during MLC are detected (Fig 13-9). This test involves an initial one-way MLC culture followed by exposure of stimulated cells to ^{51}Cr-labeled target cells specifically lysed by sensitized killer lymphocytes. These target cells are HLA-identical to the stimulator cells in MLC. Cytotoxicity is measured as the percentage of ^{51}Cr released in specific target cells compared with the percentage of ^{51}Cr released from control (nonspecific) target cells. Several lines of evidence indicate that cells which proliferate in MLC and killer cells which participate

in CML assay are not identical. Killer cells are generated that have specificity for class I MHC antigens on target cells, whereas in class II MHC antigen differences determine the reaction. CML assays provide an additional measure of T cell function and can be used to estimate presensitization and histocompatibility in clinical transplantation (see also Chapter 16).

CLINICAL APPLICATION OF T & B CELL ASSAYS

Counting of T and B cells in peripheral blood and tissue specimens has limited application in both the diagnosis and investigation of pathophysiologic mechanisms of many disease states. Functional assays are even more limited in value primarily to studies of immune deficiency diseases. Current applications include the following.

(1) Diagnosis and classification of immunodeficiency diseases (see Chapters 18–22 and 52).

(2) Determination of origin of malignant lymphocytes in lymphocytic leukemia and lymphoma (see Chapter 45).

(3) Evaluation of immunocompetence and mechanisms of tissue damage in autoimmune disease, eg, systemic lupus erythematosus and rheumatoid arthritis (see Chapter 31).

(4) Detection of changes in cellular immune competence in HIV and other infections that may be of prognostic value (see Chapter 52).

(5) Monitoring of cellular changes following organ transplantation (see Chapter 57).

NATURAL KILLER (NK) CELLS

Natural killer (NK) cells can be enumerated by specific monoclonal antibodies using methods identical to those for T and B cells (see Chapter 17). Several monoclonal antibodies are available that detect either Fc receptors (CD16) or specific differentiation antigens (CD56, CD57) present on these cells. Some NK cells also express antigens from the CD2 T cell family. Functional testing is done by measuring the ability of these nonimmune cells to kill special target cells such as erythroleukemia cell line K562. Cytotoxicity is usually performed by using the ^{51}Cr release assay, similarly to cell-mediated lympholysis (Fig 13–9).

MONOCYTE-MACROPHAGE ASSAYS

The morphologic identification of normal peripheral blood monocytes in stained peripheral blood films ordinarily is quite simple. Monocytes are larger than granulocytes and most lymphocytes. They typically have round or kidney-shaped nuclei with fine, lightly stained granules. However, in suspension or even in tissue or blood specimens, additional markers may be required to differentiate monocytes from lymphocytes and primitive myeloid cells.

A reliable stain for monocytes is so-called nonspecific esterase, or α-naphthol esterase, which is present in monocytes but absent in most myeloid and lymphocytic cells. Monoclonal antibodies directed at specific differentiation antigens such as CD14 are available.

Functional attributes of monocytes are discussed in detail in Chapter 1. In the clinical laboratory, phagocytosis of particles or antibody-coated heat-killed microorganisms is useful for functional identification of monocytes.

NEUTROPHIL FUNCTION

Polymorphonuclear neutrophils (PMN) are bone marrow-derived leukocytes with a finite life span, which play a central role in defense of the host against infection. For many types of infections, the neutrophil plays the primary role as an effector or killer cell. However, in the bloodstream and extravascular spaces, neutrophils exert their antimicrobial effects through a complex interaction with antibody, complement, and chemotactic factors. Thus, in assessing neutrophil function, one cannot view the cell as an independent entity; its essential dependence on other immune processes, both cellular and humoral, must be taken into account.

Defects in neutrophil function can be classified as quantitative or qualitative. In quantitative disorders, the total number of normally functioning neutrophils is reduced below a critical level, allowing infection to ensue. Drug-induced and idiopathic neutropenia (see Chapter 33), with absolute circulating granulocyte counts of less than 1000/μL, are examples of this sort of defect. In these situations, granulocytes are functionally normal but are present in insufficient numbers to maintain an adequate defense against infection. In qualitative neutrophilic disorders, the total number of circulating PMN is either normal or sometimes actually elevated, but the cells fail to exert their normal microbicidal functions. Chronic granulomatous disease is an example of this type of disorder (see Chapter 22). In patients with chronic granulomatous disease the normal or increased numbers of circulating neutrophils are unable to kill certain types of intracellular organisms.

Phagocytosis by PMN can be divided into 5 dis-

CELLULAR ASSAYS
FOR HISTOCOMPATIBILITY

In vivo, recognition of nonself antigens and destruction of cells bearing such markers is accomplished by cells of the immune system. Some of the clinically relevant class II HLA antigens that can trigger the immune response are not readily detected by the serologic methods discussed above. Instead, lymphocytes are used as discriminatory reagents for the HLA-Dw and -DP antigens and as indicators of histoincompatibility between donor and recipient. The functions of cellular recognition are utilized in the MLC, HTC, and primed lymphocyte typing (PLT) tests, and the dual functions of recognition and effector cell killing are used in the CML test.

MLC Test (See Chapter 13)

This is also known as the mixed-lymphocyte reaction (MLR). When the lymphocytes of 2 HLA-disparate individuals are combined in tissue culture, the cells enlarge, synthesize DNA, and proliferate, whereas HLA-identical cells remain quiescent. The proliferation is driven primarily by differences in the class II HLA antigens between the 2 test cells.

On the basis of MLC testing, class II antigens were originally described as a series of lymphocyte-activating determinants, products of the HLA-"D" locus (-Dw1, -Dw2, etc). No D locus products have ever been isolated, however, although several distinct "D region" loci (-DR, -DQ, and -DP) and their alleles have been identified. Dw "antigens" are now considered to be immunogenic epitopes formed by combinations of D region determinants that can be recognized by T cells. Distinct Dw types may represent unique haplotype combinations of various D region products.

Reactivity in MLC probably reflects the initial immune recognition step of graft rejection in vivo. The more immunogenic the D locus difference, the greater the cellular response in MLC and the more likely the rejection of the graft. Normally, both cells will proliferate, forming the 2-way MLC. To monitor the response of a single responder cell (the one-way MLC), the partner cell (stimulator) is inactivated by radiation or drugs (such as mitomycin C) that inhibit DNA synthesis (Fig 16–4). A maximum proliferative response usually occurs after incubation at 37 °C for 5–6 days. The culture is then pulsed with [³H]thymidine for 5–12 hours to label the newly synthesized DNA. Finally, the cells are harvested, washed free of unbound radioactivity, and counted in a beta counter.

A properly composed MLC test includes a checkerboard of one-way combinations of each cell serving as both stimulator and responder with all other cells. Each cell must be controlled for its ability to both stimulate and respond to HLA-mismatched cells. Normally, 2–4 unrelated control cells of known class II HLA type are tested individually with each family member. The maximum response of each cell is obtained by exposure to a pool of irradiated stimulator cells of diverse HLA types.

Autologous controls combining self with irradiated self are also run to normalize the response of each cell to stimulators. Each test should be run in triplicate. It is absolutely necessary to perform the entire familial MLC at one time owing to the inherent variability of individual cellular responses from day to day.

Results are expressed as a stimulation index (SI) or relative response (RR). The SI is the ratio of counts per minute of the test over the autologous test for that cell:

$$\text{SI} = \text{cpm of } \frac{\text{Responder vs Stimulator (irradiated)}}{\text{Responder vs Responder (irradiated)}}$$

A value of SI ≤ 2 is interpreted as HLA identity at HLA-D. The RR calculates the response in the experimental MLC relative to the maximum response of that cell elicited by the pool. Counts per minute of both are corrected by subtraction of the autologous control for the responder cell.

Figure 16–4. The MLC and CML tests. In the one-way MLC, responder PBLs are mixed 1:1 with irradiated stimulator cells and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 5 days, the culture is pulsed with [³H]thymidine ([³H]TdR) to label the nucleic acid in the responder cells. After 18 hours, cells are harvested and counted for internalized radioactivity. If the class II HLA antigens of the stimulator cells differ from those of the responder cells, the responder cells undergo blastogenesis, synthesize DNA, and proliferate. Increased sample radioactivity signals recognition of class II HLA differences. When responder and stimulator cells are class II identical, the proliferative responses are less than 20% of the maximum response to the mismatched controls and less than 2% over autologous (background) controls. (Reproduced, with permission, from Bach FH, Van Rood JJ: The major histocompatibility complex: Genetics and biology. *N Engl J Med* 1976;**295**:806, 872.)

$$RR = \frac{\text{cpm of} \quad \frac{\text{Responder vs Stimulator (Irradiated)} - \text{Autologous control}}{\text{Responder vs Pool (Irradiated)} - \text{Autologous control}}}{} \times 100$$

MLC testing can be useful in selecting the most compatible (least stimulatory) organ donor if several nonidentical family members (matched for zero or one haplotype) are available. The donor who is the least stimulatory to the patient is the preferred organ donor. The results of an intrafamilial MLC can address such questions as (1) Are 2 serologically identical DR antigens also functionally identical? (2) Is the individual with only one identifiable DR antigen a homozygote, or is the DR "blank" really a second DR antigen that was missed in the serologic testing? (3) Are the serologically assigned class II antigens consistent with the MLC results? If apparently HLA-identical individuals are reactive, there may have been a genetic recombination event.

The MLC test is frequently used to confirm apparent HLA identity in the living-related transplant situation and is especially useful if haplotyping could not be accomplished. When the HLA-identical recipient and donor are unrelated, as in voluntary bone marrow donation, MLC testing is extremely important in revealing hidden class II incompatibilities that could affect recipient tolerance to the graft and promote graft-versus-host disease.

HTC Test

MLC nonreactivity indicates HLA-Dw identity, and therefore the MLC test can be used to type for specific Dw alleles. Since most individuals to be tested are heterozygous at the D region, it follows that the stimulator cell must be homozygous for a given Dw antigen to result in MLC nonresponsiveness. Such HTCs have been identified in the random population, but the best source for HTCs is among the progeny of first-cousin marriages. True HTCs are rare and precious reagents, and few laboratories can afford to maintain the large cell panel necessary for complete and accurate testing.

The lymphocytes to be Dw typed are set up as responders in multiple MLC tests, each test with a different inactivated stimulator HTC. HTC testing can discriminate among the various subtypes of serologically identified DR antigens and thus can provide a finer definition of true class II HLA compatibility.

PLT Test

Lymphocytes already exposed (primed) to a specific antigen in a primary MLC will proliferate rapidly on reexposure to the same antigen. Thus, a primed cell can be used to test an unknown cell for the presence of the original stimulating antigen. With cells primed to class II HLA antigens, this assay can be used as an HLA typing test for D region antigens.

As in HTC typing, an extensive panel of specifically primed cells must be maintained for PLT testing. PLT was used to type for DP antigens, but this method has been supplanted by molecular typing methods and is now only rarely used.

CML Test

In primary MLC, exposure to nonself class I and class MHC II antigens can result in the generation of cytotoxic T lymphocytes (CTL). CTL kill their targets through direct contact, probably by the release of toxic mediators that lead to cell lysis. CD4 and CD8 CTL can be found infiltrating kidney allografts during rejection and are considered to be important effector cells in graft loss (see Chapter 57). To test for the capacity to generate CTL, a primary MLC is run with the patient as the responder and prospective donor cells as inactivated stimulators. After the MLC, the patient's cells are harvested and then reexposed in culture to fresh donor target cells that have been loaded with ^{51}Cr (Fig 16–4). Usually, the targets are preincubated with the mitogen phytohemagglutinin (PHA) for 6 days, since PHA-activated blast cells can incorporate more ^{51}Cr than resting lymphocytes can. CTL and targets are plated in effector:target-cell ratios of 100:1, 50:1, and 10:1. Control wells include targets alone to measure the spontaneous release of label and test wells containing target cells that are treated with detergent to release the maximum incorporated label. The test requires 4 hours of incubation in a humidified CO_2 atmosphere at 37 °C. At the conclusion, the supernatant of each test well is sampled and counted. In the experimental wells, the amount of ^{51}Cr released is corrected for the background level of spontaneously released label and compared with the maximum amount of label released:

$$\% \text{ Specific Release} = \frac{\text{cpm (Experimental)} - \text{cpm (Spontaneous)}}{\text{cpm (Maximum)} - \text{cpm (Spontaneous)}} \times 100$$

Elevated counts of 30–50% above spontaneous background are indicative of CTL activity.

Direct CML testing can be used to monitor posttransplant rejection by testing for the presence of activated circulating anti-donor CTL. The patient's PBL are placed directly in culture with ^{51}Cr-labeled donor cells as targets. An elevated donor cell lysis compared with pretransplant levels is considered evidence of circulating CTL, which are particularly prevalent during rejection.

CML testing has applications in living-related renal and bone marrow transplantation. The preferred kidney donor will be the one who fails to stimulate the recipient to form CTL. In bone marrow transplantation, the recipient is at risk for immune attack by the

Table 20–1. Evaluation of cell-mediated immunity.

Test	Comment
Total lymphocyte count	Normal at any age: >1200/μL.
Delayed hypersensitivity skin test	Used to evaluate specific immunity to antigens. Suggested antigens are *Candida*, mumps, purified protein derivative, and streptokinase-streptodornase (4 units/0.1 mL).
Lymphocyte response to mitogens (PHA), antigens, and allogeneic cells (mixed-lymphocyte culture)	Used to evaluate T cell function. Results are expressed as stimulated counts divided by resting counts (stimulated index).
Total T cells using monoclonal antibodies to CD3, CD2, or CD4 plus CD8	Used to quantitate the number of circulating T cells. Normal: >60% of total lymphocytes.
Monoclonal antibody to T cells and T cell subsets (CD4 and CD8)	Determines total number of T cells as well as T cell subsets, eg, helper/suppressor.
Cytokine production (IL-1, IL-2, lymphotoxin, tumor necrosis factor, etc)	Used to detect specific cytokine production from subsets of mononuclear cells as an index of function.
Helper/suppressor T cell function	Provides information on T cell regulation of immunity.

Figure 20–1. Infant with DiGeorge's syndrome. Prominent are low-set and malformed ears, hypertelorism, and fish-shaped mouth. Also note the surgical scar from cardiac surgery.

Clinical Features

A. Symptoms & Signs: The most frequent presenting sign in patients with DiGeorge's syndrome occurs in the first 24 hours of life with hypocalcemia that is resistant to standard therapy. Various types of congenital heart disease have been described, including interrupted aortic arch, septal defects, patent ductus arteriosus, and truncus arteriosus. Renal abnormalities may also be present. Some patients have the characteristic facial appearance described above. Patients who survive the immediate neonatal period may then develop recurrent or chronic infection with various viral, bacterial, fungal, or protozoal organisms. Pneumonia, chronic infection of the mucous membranes with *Candida,* diarrhea, and failure to thrive may be present.

Spontaneous improvement of T cell immunity occasionally occurs. These patients are considered to have "partial" DiGeorge's syndrome, but the reason for the spontaneous improvement in T cell immunity is not known. Patients have also been suspected of having DiGeorge's syndrome on the basis of hypocalcemia and congenital heart disease with or without the abnormal facies but have been found to have normal T cell immunity. Subsequently, these patients may develop severe T cell deficiency.

B. Laboratory Findings: Evaluation of T cell immunity can be performed immediately after birth in a patient suspected of having DiGeorge's syndrome. The lymphocyte count is usually low (<1200/μL) but may be normal or elevated. In the absence of stress during the newborn period, a lateral-view x-ray of the anterior mediastinum may reveal absence of the thymic shadow, indicating failure of normal development. Delayed hypersensitivity skin tests to recall antigens are of little value during early infancy, because sufficient time has not elapsed for sensitization to occur. T cells are markedly diminished in number, and the peripheral blood lymphocytes fail to respond to phytohemagglutinin (PHA) and allogeneic cells.

Studies of antibody-mediated immunity in early infancy are not helpful, because immunoglobulins consist primarily of passively transferred maternal IgG. Although it is believed that some of these patients have a normal ability to produce specific antibody, the majority have some impairment of antibody formation. Sequential studies of both T cell and B cell immunity are necessary, since spontaneous remissions and spontaneous deterioration of immunity with time have been described.

A diagnosis of hypoparathyroidism is established by the demonstration of low serum calcium levels, elevated serum phosphorus levels, and an absence of parathyroid hormone. Congenital heart disease may be diagnosed immediately following birth and may be mild or severe. Other congenital abnormalities in-

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Evaluation of the Cellular Immune Response in Transplantation

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Solid-organ transplantation has become an increasingly important therapeutic modality for patients with various end-stage diseases. Despite improved immunosuppression protocols, most transplant recipients face a variety of complications. Early after transplant, infection and rejection are the major causes of morbidity and mortality. Drug toxicity, chronic rejection, and malignancies are long-term complications.

Many attempts have been made to develop in vitro procedures that can assess the immunologic status of the allograft reliably and accurately. Immunological monitoring could, in theory, differentiate rejection from other forms of dysfunction, such as infection or primary nonfunction. Furthermore, the ideal tool would be able to gauge accurately a patient's response to antirejection therapy and might help prevent overimmunosuppression. Immune monitoring could also be important in predicting long-term graft outcome and thereby identify which recipients could have their immunosuppression markedly reduced without increasing the risk of acute or chronic rejection.

Although there is no one ideal immunological test that can accomplish all of the above, there are a number of tests that can be used together to assess the immunological status of the transplant recipient. The most informative approach appears to be performing a sequential analysis using assays that measure different immune functions.

MLC

Concept

Mixed leukocyte culture (MLC) is perhaps the most widely used cellular assay. It represents a functional assay of cellular response to stimulatory determinants associated predominantly with HLA class II molecules, including HLA-DR, -DQ, and, to a lesser extent, HLA-DP. The first descriptions of this assay as a measurement of cellular immunity (3, 4), together with the development of a one-way method of stimulation, allowed the correlation of proliferative responses between siblings. The conclusion was that a single genetic locus or region, now known as HLA, controlled the MLC reactivity. The recognition of disparate HLA class II molecules and the resulting T-cell activation (as measured in MLC) are thought to represent an in vitro model of the afferent arm of the in vivo allograft reaction.

MLC is an in vitro test of lymphocytes responding to stimulation by disparate HLA class II molecules, which are predominantly expressed on B cells and monocytes of the stimulator cell population. Proliferative reactivity to HLA class I molecules has been reported but plays a minimal role in the overall bulk MLC response. In MLC, stimulator cells have been inactivated, usually by X-irradiation, and can no longer divide. The resulting proliferation of responding cells involves the logarithmic expansion of multiple clones of alloactivated T cells. This expansion can be measured by incorporation of the radioisotope-tritiated thymidine ([³H]thymidine) into replicating DNA during the logarithmic phase of cellular expansion, usually on the fifth day of culture. The amount of thymidine incorporated into cellular DNA is then assayed by liquid scintillation spectrophotometry. Exogenous [³H]thymidine added to in vitro cultures is incorporated during DNA replication via the salvage pathway, in which free purine bases are formed by hydrolytic degradation of nucleic acid and nucleotides. Exogenous [³H]thymidine is added to cultures for a period of time that is longer than the S phase of the cell cycle but shorter than the cell cycle itself, usually 18 h.

The degree of reactivity observed correlates with the degree of antigenic disparity between responding and stimulating cells. MLC has been used clinically for donor selection, predominantly for bone marrow transplantation. With the more recent application of DNA-based HLA typing methods, MLC is used less often for donor selection but more often for following the recipient's posttransplant donor antigen-specific immune status. The difference between posttransplant and pretransplant antidonor MLC responses can be used to define any changes (increases or decreases). Studies have shown that solid-organ recipients who develop a decreased response (i.e., are hyporesponsive) are at low risk for immunologic complications, such as late acute rejection episodes and chronic rejection. Hyporeactivity is defined as at least a 60% decrease in reactivity of the posttransplant antidonor response compared with the pretransplant antidonor response, assuming the response to third-party cells remains unchanged (26, 27).

Procedure

Sample Requirements

Collection of specimens. Care must be taken throughout the procedure to ensure a sterile specimen. Usually, 20 to 30 ml of sterile heparinized blood is obtained from the blood donor. The specimen may be saved overnight but should be processed within 24 h of the phlebotomy. It should be maintained at room temperature even if it is being shipped by overnight carrier. Poor cell yields may result from temperature conditions that are too cold or too warm.

Materials and Reagents

Lymphocyte separation medium (LSM) (Pharmacia Biotechnologies, catalog no. 17084003)

Culture medium: RPMI 1640 with HEPES buffer supplemented with 100 U of penicillin per ml, 100 U of streptomycin (Grand Island Biological, catalog no. 380-2400AJ) per ml, 10 U of preservative-free heparin (Monoparin heparin; Accurate Chemical & Scientific Corp., catalog no. A6500) per ml, 2 mM L-glutamine, and 10 to 20% pooled human sera (PHS)

PHS: serum from 10 to 20 healthy nontransfused male donors, heat inactivated at 56°C for 30 mm (see "Pitfalls and Troubleshooting" below for additional specifications)

[³H]Thymidine: specific activity of 6.7 Ci/mM; 0.5 to 1.0 mCi per well is commonly used (New England Nuclear, catalog no. NEN-027)

Hanks' balanced salt solution (Grand Island Biological, catalog no. 310-4170PJ)

Equipment and Instrumentation

Radiation source (usually gamma-emitting radiation source); alternatively, mitomycin (Sigma, catalog no. M0503)

Laminar flow hoods (Baker 60; The Baker Co., Sanford, Maine)

Liquid scintillation counter (1205 Betaplate; Pharmacia LKB)

Rate freezer (model 70014; CryoMed)

Liquid nitrogen refrigeration unit (model CAIIIL; CryoMed)

Mechanics and Controls

Mononuclear cells are isolated by centrifugation of peripheral blood diluted 1:2 with Hanks' balanced salt solution over LSM. Peripheral blood mononuclear cells (PBMC) are removed from the LSM interface, diluted with Hanks' balanced salt solution, and then centrifuged at $500 \times g$ for 10 min. The supernatant is decanted, and the wash steps are repeated two more times. The cells are resuspended in an exact quantity of complete culture medium. A leukocyte count is done, and viability is determined via dye exclusion. The cell suspension is diluted to a final concentration of 5×10^5 PBMC per ml, using the culture medium. Stimulator cells are inactivated either by irradiation at 1,500 to 3,000 rads or by incubation with mitomycin according to the manufacturer's instructions. With a repeating microliter pipette, stimulator and responder cells are added in triplicate to round-bottom microtiter plates (ICN, catalog no. 760-042-05), such that each well receives 100 μl of stimulator cells (5×10^4 PBMC) and 100 μl of responding cells (5×10^4 PBMC).

A complete culture setup includes the following:

- Allogeneic cultures containing all possible combinations of responder and stimulator cells, including cells from three control cell donors of a known HLA phenotype
- Autologous cultures containing the responder and stimulator cells from the same cell donor
- Control wells containing either responder or stimulator cells alone, with an equal volume of complete culture medium
- Double irradiation control cultures containing stimulator cells from two different cell donors

The cultures are incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days, after which 0.5 to 1.0 μCi of [³H]thymidine is added to each well. The cultures are incubated for an additional 18 h. The culture plates can then be harvested immediately or sealed with pressure-sensitive film and placed in the refrigerator until harvesting. A number of different harvesting machines and counting systems are available: the cells can be harvested onto filter disc sheets, or the samples can be counted in vials or cassettes or directly, without the need for scintillation fluid. The manufacturer's instruction manual should describe the appropriate procedures.

Pitfalls and Troubleshooting

- *Drugs*. If a patient is taking one of the following drugs, the proliferative response may be compromised: prednisone, Myleran, hydroxyurea, Cytoxan, or L-asparaginase.
- *Serum*. One of the most common sources of technical problems in any cellular procedure is a poor serum source. Each individual lot of a serum source or, preferably, each individual serum unit within the lot should be screened for growth support capabilities and possible HLA antibodies. The screen should include a control response to a pool of allogeneic cells to measure maximum response and an autologous control to ensure low backgrounds. If sporadic high backgrounds are observed, an endotoxin test may be advisable.
- [³H]*Thymidine*. If low counts per minute are observed, the scintillation counter and the shelf life of the [³H]thymidine should be checked. The half-life of the ³H is 12.3 years, but the shelf life of the thymidine is considerably shorter.
- *Frozen cells*. Cells to be used as responder cells in the cell cultures can be bulk frozen by a step-down procedure at 4, −30, and −70°C before use. However, viability and cell recovery are better if the cells are rate frozen and stored in the vapor phase of a liquid nitrogen storage unit.

The *American Society for Histocompatibility and Immunogenetics Procedure Manual* (28) is an excellent source of additional information and details on cellular methods.

Interpretation

Results are usually expressed as raw counts per minute of [³H]thymidine incorporation. The data may be reduced to

allow for easier interpretation and comparability from one test to another. The two most common forms of data reduction are the stimulation index and the RR. The stimulation index is a simple ratio of the counts per minute from an experimental MLC combination to the counts per minute of the autologous control. The RR is the ratio of the net counts per minute (after subtraction of the autologous control counts per minute of an allogeneic MLC combination) to that of a maximally stimulated or control MLC combination (usually the response to a pool of allogeneic cells), multiplied by 100 to obtain a percentage (28).

VARIATIONS

MTT Method

A nonradioactive alternative to detecting proliferation is a colorimetric (3-4,5-dimethylthiazol-2-yl)-2.5 diphenyl tetrazolium bromide salt (MTT) reduction assay. This assay detects the function of a mitochondrial enzyme that reflects the number of metabolically active cells present in the culture. The MTT, a yellow aqueous solution, is taken up by the viable cells and reduced in the mitochondria to a purple crystal. After solubilization of these crystals, the reaction is measured with a spectrophotometer. An increase in the measured optical density parallels an increase in mitochondrial enzyme activity, which reflects an increase in the number of cells. This approach can detect increases in an interleukin-2 (IL-2)-sensitive line, CTLL2O. Van Buskirk et al. (36) have demonstrated that colorimetric detection of IL-2 production correlates well with the radioactive detection of T-cell proliferation and can be used interchangeably with the standard MLC.

Multiparameter Flow Cytometry

A second nonradioactive method to detect T-cell activation is a flow cytometry assay. It detects an early activation antigen, CD69, which reaches peak expression within 8 h of stimulation (17). The assay uses a three-color technique to detect CD3 CD69 CD4$^+$ or CD3 CD69 CD8$^+$ cells. Advantages are short activation time (4 h), absence of radioactivity, avoidance of peripheral blood lymphocyte separation, ability to identify activated T-cell subsets, quick turnaround (results can be obtained in hours), simplicity, and high sensitivity (17). This technique may be especially informative when analyzing mitogen or antigen stimuli; however, analyzing a response to allogeneic cell stimulus may be more difficult (FastImmune Assay System; Becton Dickinson).

T-CELL-PRECURSOR FREQUENCY DETERMINATION BY LDA

The frequency of T-cell precursors is determined by limiting dilution assays (LDA). Limiting numbers of responder cells are cultured with a constant number of stimulator cells and assayed for reactivity (cytotoxic, proliferative, or cytokine release) against additional stimulator cells. In contrast to MLC, which measures a bulk response, LDA is a quantitative tool. It allows the investigator to estimate the frequency of lymphocytes with a given function and antigen specificity. LDA imply that the lysis is a single-hit process; that is, a single precursor cell will initiate the sequence of events that leads, in the case of cytotoxic precursors, to the eventual lysis of the cell. A frequency can be determined if the lymphocyte population is random;

that is, the function of the lymphocyte population is not influenced by the presence of another lymphocyte population. The clear distinction between a response and a lack of that response is imperative. Making this distinction is more difficult in the cytotoxicity assay, in which an arbitrary threshold that separates the spontaneous release of ^{51}Cr from the release from the lysed targets is set. The frequency of responding cells is determined by a maximum likelihood estimation, using a computer program (13).

LDA of HTL

Helper T lymphocytes (HTL) are detected by their ability to produce IL-2. The murine IL-2-dependent line, CTLL2O, is used as the indicator line in this bioassay. Adding CTLL2O cells directly to the micrometer wells is more sensitive than removing an aliquot of supernatant and adding it to the CTLL2O cells. Before adding the indicator line, the plates are irradiated to inhibit the responder cells from proliferating and incorporating [^{1}H]thymidine. A low dose of radiation does not block the continued production of IL-2 by the responding cells. In the absence of IL-2, the CTLL2O cells die rapidly. Therefore, any proliferation detected is due to division of the IL-2-stimulated CTLL2O cells.

Method

LDA can be done on fresh or cryopreserved cells. The cells are prepared as described in the section on the MLC protocol. Limiting numbers of PBMC (2 × 10^4, 1.0 × 10^4, 0.5 × 10^4, 0.25 × 10^4, 0.125 × 10^4, 0.0625 × 10^4, and 0.03125 × 10^4) are cultured in round-bottom microtiter plates with constant numbers (10 × 10^4) of irradiated (3,000 rads) stimulator cells. When testing HLA-identical pairs, increased numbers of cells are often used, such that the highest concentrations are 4 × 10^4, 6 × 10^4, 8 × 10^4, or 10 × 10^4 cells. Multiple wells per dilution are necessary to ensure an accurate assessment of the frequency. Usually, 24 wells per dilution are set up. The culture medium is the same as that used in the MLC protocol. Control wells consist of 24 wells containing irradiated stimulator cells alone (for calculation of background activity), responder cells in medium alone (negative controls), and responder and stimulator cells set up separately against HLA-mismatched third-party cells (positive controls). The plates are incubated for 64 h at 37°C in a 5% CO$_2$ environment. The plates are irradiated with 2,500 rads. CTLL2O cells (1 × 10^4) are added in 25 ml of medium. The plates are incubated for 8 h with 1 mCi of [^{1}H]thymidine in 25 ml per well. Cultures are reincubated for 16 h. Cultures are harvested and counted as outlined in the MLC protocol. The wells are considered positive if [^{1}H]thymidine incorporation is greater than the mean plus 3 standard deviations of the 24 control wells. The frequency of responding cells is determined by a maximum likelihood estimation, using a computer program, and the variance is determined by the use of 95% confidence intervals. Regression analysis is used to generate a straight line. Chi-square analysis is used to show that the data obtained are in accordance with single-hit kinetics. A program that does all the necessary calculations for this analysis is described in reference 13.

Pitfalls and Troubleshooting

The sensitivity of this assay depends on the condition of the IL-2 indicator cells. The proliferation of the CTLL2O line depends on murine or human IL-2 or murine IL-4, without which the cells will die rapidly. The line should be maintained at a concentration of 1 × 10^4 to 10 × 10^4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Docket No.:

Serial No.:   Group Art Unit:

Filing Date: Examiner:

For:

DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.

2. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.

3. My scientific Curriculum Vitae is attached to and forms part of this Declaration.

4. I am familiar with the Mixed Lymphocyte Reaction (MLR) assay, which has been used by me and others under my supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.

5. The MLR assay is a well known and widely used proliferative assay of T-cell function, the basic protocols of which are described, for example, in Current Protocols in Immunology Vol. 1, Richard Coico, Series Ed., JohnWiley & Sons, Inc., 1991, Unit 3.12. (Exhibit A). This publication is incorporated by reference in the description of the MLR protocol in the present application.

6. The T-lymphocytes or "T-cells" of our immune system can be induced to proliferate by a variety of agents. The MLR assay is designed to study a particularly important induction mechanism whereby responsive T-cells are cultured together (or "mixed"), with other lymphocytes that are "allogeneic", e.g. lymphocytes that are taken from different individuals of the same species. In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells. It is known that the dendritic cell population among the PBMCs are differentially affected by irradiation. At low doses (500-1000 Rad), the proliferation of most cells, including the B cells in the PBMCs, is preserved, however, at doses above 2000 Rad, this function of B cells is abolished. Dendritic cells on the other hand, maintain their antigen presentation function even at a 3000 Rad dose of radiation. (See, e.g. Current Protocols in Immunology, supra, at 3.12.9). Accordingly, under the conditions of the MLR assay used to test the PRO polypeptides of the present invention, the stimulator PBMCs remaining after irradiation are essentially dendritic cells.

7. Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naive T cells in vivo. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells. For further details about the properties and role of dendritic cells in immune-based therapies see, e.g. Steinman, Drug News Perspect. 13(10):581-586 (Exhibit B).

8. The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

9. Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured in vitro and returned to the patient to stimulate anti-cancer response. [Thurner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].

10. It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Some PRO polypeptides do the reverse, and give inhibition of T-cell proliferation in the MLR assay. It is my considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.

Dated: 6/16/04

By: _Sherman Fong_

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Education:

1978 - 1980   Postdoctoral Fellow in Immunology,   Research Institute of Scripps Clinic,
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978   Postdoctoral Fellow in Immunology,   University of California at
San Francisco, San Franciso, California

1970 - 1975   Ph.D. in Microbiology,   ,   University of California at
Davis, California

1966 - 1970   B.A. in Biology/Microbiology,   San Francisco State
University, San Francisco, California

Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc. South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Resarch Grant, NIH (1985)

Recipient: P.I., Resarch Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Commitee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmcological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the Biochemical Subteam for Protropin® Project team.

Served as Immunologist on the Met-less hGH and Dnase project teams, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in 1989-1990 on the CD4-IgG project team carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte β2 integrins. Provided preclinical scientific data to Anti-CD18 project team

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, Research Project Team leader for small molecule α4β1 integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped established and responsible for maintaining current research collaboration with Roche-Nutley. Project transfered to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against α4β7 intergin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn';s disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, Research Project Team Biology Leader (1996-present) for small molecule antagonists for α4β7/MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as Ad Hoc reveiwer on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as Scientific advisor with staff of the Business Development Office on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001   Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The β2 Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process -- Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process -- Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin α4β7 Attentuates Inflammatory Bowel Diseases", in Sympoium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.
U.S. Patent Number 5,068,177: Anti-idiotype Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.
U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.
U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colits, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen
U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood
U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood . U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura
US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood
US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

Publications:

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2. Scibienski R, Harris M, Fong S, Benjamini E: Active and inactive states of immunological unresponsiveness. J Immunol 113:45-50, 1974.

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17. Pasquali J-L, Fong S, Tsoukas CD, Hench PK, Vaughan JH, Carson DA: Selective lymphocyte deficiency in seronegative rheumatoid arthritis. Arthritis Rheum 24:770-773, 1981.

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CURRENT PROTOCOLS IN IMMUNOLOGY

VOLUME 1

Proliferative Assays for T Cell Function

UNIT 3.12

A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (*UNIT 3.10*) or cytotoxicity (*UNIT 3.11*)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (*UNIT 3.13*) or T cell hybridomas (*UNIT 3.14*).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. The first basic protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR-$\gamma\delta$, or anti-TCR-$\alpha\beta$ monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the *V* regions of β chains of the TCR (anti-Vβ) or with enterotoxins specific for certain Vβ-chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). The first alternate protocol describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. The second alternate protocol describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). The first support protocol describes the preparation and use of T cell–depleted accessory or stimulator cells and the second support protocol describes methods for blocking accessory cell proliferation. Finally, the second basic protocol describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed in vivo.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in *UNIT 3.10*. Related assays for use with human peripheral blood lymphocytes are described in *UNIT 7.9*.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

ACTIVATION OF UNPRIMED T CELLS

BASIC PROTOCOL

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T cell proliferation is dependent on the presence of non–T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non–T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell–independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [^{3}H]thymidine between stimulated and control cells.

In Vitro Assays for Mouse B and T Cell Function

Contributed by Ada M. Kruisbeek and Ethan Shevach
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3.12.1

Table 3.12.1 Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent[a]	Source/cat. no.[b]	Concentration	Accessory cells[c]	Mode of action, etc.
PMA	SIG P8139	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL 407950	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL 100105	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD HA16	1-5 µg/ml	Yes	Indirect TCR cross-linking
Con A	PH 17-0450-01	1-10 µg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 µg/ml	Yes[c]	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 µg/ml	Yes[c]	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 µg/ml	Yes[c]	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2[c]	PG MM-Vβ-TCR-1	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6[c]	PG RM-Vβ-TCR-2	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT AT101	1-10 µg/ml	Yes[c]	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT BT202; SIG S4881	1-100 µg/ml	Yes[c]	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT ET404	1-10 µg/ml	Yes[c]	Vβ-11,15,17-receptor specificity

[a]Abbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus* enterotoxins A, B, & E.

[b]Supplier addresses and phone numbers are provided in APPENDIX 5. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

[c]When using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

Materials

Complete RPMI-5 and RPMI-10 media (*APPENDIX 2*)

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNIT 3.1*)

Activating agent(s) (Table 3.12.1)

Phosphate-buffered saline (PBS; *APPENDIX 2*)

Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (second support protocol) or T cell–depleted (first support protocol)

[³H]thymidine (*APPENDIX 3*)

15- and 4-ml disposable, polystyrene conical tubes with screw caps

Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)

1-, 5-, and 10-ml disposable polystyrene pipets

96-well flat- or round-bottom microtiter plates with lids (Costar #3596 or #3799)

25- to 100-µl single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (*UNIT 1.9*), preparing single-cell suspensions (*UNIT 3.1*), and counting, labeling, and harvesting cells (*APPENDIX 3*)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in *UNIT 3.1*.

   The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4⁺ or CD8⁺) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~1000 rpm ($200 \times g$), room temperature, and discard supernatant.

3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to ~10⁶ cells/ml with complete RPMI-10.

   While this concentration (1×10^6 cells/ml or 2×10^5 cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8×10^5 cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non–T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers (0.1, 0.5, and 1.0×10^5) of syngeneic spleen (accessory) cells in 0.1 ml to 2×10^5 T cells in 0.1 ml (see first support protocol). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from 1 mg/ml stock solutions—e.g., 100, 30, 10, and 3 µg/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1 µg/ml A23187 (or 4 µg/ml ionomycin) in PBS.

   If MAb in supernatant or ascites form are being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.

See Table 3.12.1 for Vβ specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enteroxins and their specificities.

5. Add 20 μl of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20 μl of PBS only. Add 20 μl PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

   A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.

6. To the wells of the 96-well microtiter plate containing activating agent, add 2×10^5 cells in 0.2 ml.

7. Place microtiter plates in a humidified 37°C, 5% CO_2 incubator for 2 to 4 days.

   Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see critical parameters).

8. Add [^{3}H]thymidine to each well. Return the plates to CO_2 incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.

9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as "Δ cpm."

9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to "SI" (stimulation index).

   The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications, Δ cpm rather than SI values are preferred.

ALTERNATE PROTOCOL

ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor–bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in the basic protocol should be followed.

Additional Materials

PBS (APPENDIX 2), room temperature and 4°C

1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) or 1 mg/ml purified anti-Vβ or anti-TCR-γδ MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1 µg/ml—using room temperature PBS.

   Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.

   The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.

   Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FCS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.

2. Add 30 µl of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30 µl PBS only.

   A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.

   Most often, optimal responses are seen with 10 µg/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is ~2 to 3 µg (A.M.K., unpub. observ.).

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

   During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.

4. Prepare responder cell suspensions as in steps 1 to 3 of the basic protocol.

   Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell–independent. However, the presence of non–T accessory cells does not interfere with the proliferative response.

5. Wash the wells of the incubated plates by adding 200 µl cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add ~2 × 10^5 cells in 0.2 ml.

   If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100 µl PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to ≤4 day periods. The PBS should be removed before the cells are added.

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Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of the basic protocol, but incubate cultures for 2 to 3 days before adding [^{3}H]thymidine.

    Kinetic assays should be performed to determine the optimum culture period.

T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [^{3}H]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see support protocols).

Additional Materials

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (*UNITS 1.9 & 3.1*) or purified T cells or T cell subpopulations (*UNITS 3.1-3.6*)
Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at *H-2* or *Mls* loci, irradiated or treated with mitomycin C (second support protocol) or T cell–depleted (first support protocol)

1. Prepare responder cell populations as in steps 1 to 3 of the basic protocol. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

    To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following: 0.5, 1, 2, and 4 × 10^5 cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.

    For thymocytes, it may be necessary to use 8 × 10^5 cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be 1 × 10^5 and the doses in between would be 2 and 4 × 10^5. Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.

2. To a 96-well microtiter plate, add 5 × 10^4 to 4 × 10^5 responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

    Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (UNIT 3.11), which is performed in 24-well microtiter plates. For example, cells can be diluted to 4 × 10^6 cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.

3. Prepare a single-cell suspension of irradiated or mitomycin C–treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

    The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from 0.5-4 × 10^5, test stimulator cells at densities of 2, 4, and 8 × 10^6/ml (i.e., 2, 4, and 8 × 10^5/well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If

highly purified cells are used as the responder population, it is therefore not necessary to supplement the cultures with non–T accessory cells syngeneic to the responder T cells.

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C–treated syngeneic stimulator cells. Values obtained from these controls reflect "background" proliferation values (see step 9 of basic protocol). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow steps 7 to 9 of the basic protocol, but incubate the cultures for 3 to 6 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see critical parameters).

DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS

Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (second support protocol) can produce cytokines. In the following steps, T cell–depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non–T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell-depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in *UNIT 3.7* does not deplete T cells and therefore is not recommended here; the method described in *UNIT 3.15* leads to higher levels of enrichment that are not required in the protocols presented here.

Additional Materials

    Spleen cells from nonimmunized mice
    Hanks balanced salt solution (HBSS; *APPENDIX 2*)
    Low-Tox rabbit complement (Cedarlane #CL3051), reconstituted with
        ice-cold distilled water and filter-sterilized
    Anti-Thy-1.2 ascites (HO-13-4; ATCC #TIB 99) or anti-Thy-1.1 ascites
        (HO-22-1; ATCC #TIB 100; alternatively, see Table 3.4.1 for other
        anti-Thy-1 MAb and *UNIT 2.6* for production of ascites)
    70% Percoll solution (*UNIT 3.8* and reagents and solutions)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25 μl anti-Thy-1 ascites.

If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.

The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.

3. Incubate the mixture at 45 min in a 37°C water bath.

4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × g), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.

5. Count viable cells (APPENDIX 3) and resuspend in complete RPMI-10 or PBS for inactivation as in the second support protocol, or in HBSS to prepare low-density accessory cells (see below).

   The T cell–depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell–depleted spleen cells from step 5 in HBSS at 20 × 10⁶ cells/ml.

7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.

8. Spin 13 min in H-1000B rotor at 3000 rpm (1900 × g), room temperature.

9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.

10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to the second support protocol.

    The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.

BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in the basic protocol, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see APPENDIX 1, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by g irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

Mitomycin C Treatment

Additional Materials

   Mitomycin C (Sigma #M-0503; store in dark)

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1. In a 15-ml aluminum foil–wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

   Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.

2. Prepare spleen cell suspension as described in steps 1 and 2 of the basic protocol at a concentration of 5×10^7 cells/ml in PBS.

3. Add mitomycin C to a final concentration of 50 μg/ml (100 μl/ml of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.

4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm ($300 \times g$). Discard supernatant and repeat washing procedure two more times.

   Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.

5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to step 6 of the basic protocol.

Irradiation Treatment

Prepare a spleen cell suspension as described in steps 1 to 3 of the basic protocol, at a final concentration of 5-10×10^6 cells/ml in complete RPMI-10. Using a source of ionizing irradiation (^{60}Co or ^{137}Cs γ-irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses >2000 rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of <1000 rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

ACTIVATION OF PRIMED T CELLS

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require in vivo immunization followed by in vitro stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary in vitro responses (i.e., MHC antigens) can be obtained by in vivo priming. Multiple immunizations usually elevate in vitro responses.

To immunize animals for in vitro secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freunds adjuvant (*UNIT 2.5*). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For

strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with $1-5 \times 10^7$ cells that express the antigen. Immunization protocols are described in *UNIT 1.6*.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (*UNIT 3.14*) and T cell hybridoma preparation (*UNIT 3.13*).

Materials

Complete RPMI-10 medium (*APPENDIX 2*)
Responder cells: Purified T cells isolated from lymph nodes (*UNITS 3.1-3.6*) of in vivo primed mice
Antigen: 1 mg/ml sterile protein antigen(s) (*UNIT 3.13*), in PBS *or* suspension of irradiated or mitomycin C–treated stimulator cells expressing alloantigens at 8×10^6 cells/ml (*UNIT 3.11*, support protocol) in complete RPMI-10 medium (*APPENDIX 2*)
Accessory cells: suspension of irradiated or mitomycin C–treated (or T cell–depleted) spleen cells syngeneic to the responding T cells at 5×10^6 cells/ml in complete RPMI-10 medium

4-ml conical tubes
96-well flat-bottom microtiter plates with lids

1. Follow steps 1 to 3 of the first basic protocol for preparation of responder cells.

2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

   The following dilutions are recommended: 100, 10, 1, and 0.1 µg/ml protein antigens and 8, 4, 2, and 1×10^6 cells/ml of stimulator cells in complete medium.

3. Add antigens to 96-well flat-bottom microtiter plates, at 30 µl/well for protein antigens or 100 µl/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

   By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.

4. Add responder T cells in 0.1 ml to each well.

   Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non–T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add $1-2 \times 10^5$ cells per well and proceed to step 6.

5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at 5×10^5 cells per well.

   Purified T cells require an exogenous source of accessory non–T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.

6. Proceed as in steps 7 to 9 of the basic protocol.

   Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.

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REAGENTS AND SOLUTIONS

Percoll solution

Diluent:
45 ml 10× PBS, pH 7.4 (*APPENDIX 2*)
3 ml 0.6 M HCl
132 ml H_2O
Filter sterilize

70% Percoll solution:
63 ml Percoll (Pharmacia LKB #170891-01)
37 ml sterile diluent (above)
Final osmolarity should be 310 to 320 osM

COMMENTARY

Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in *UNIT 3.11* or the lymphokine production assays in *UNITS 3.15 & 6.3*. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variously reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "bystander" cells, and will be severely affected by the function of non-T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in *UNITS 3.15 & 6.3*. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (*UNIT 6.1*) or by flow cytometry (*UNIT 5.4*). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

Critical Parameters and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO_2 level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in the first basic protocol to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR-$\alpha\beta$ or -$\gamma\delta$, as well as anti-Vβ antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (*V*) gene segment of the TCR. Different toxins have affinities for different Vβ chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the

mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (UNIT 3.15). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or "second signals" are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of UNITS 3.8, 3.11, & 3.13. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C-treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see first support protocol) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [^{3}H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and "cold" thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [^{3}H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [^{3}H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [^{3}H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal calf serum (FCS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. Our laboratory has found consistently better responses in round-bottom plates when

thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [3H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in APPENDIX 2), presumably because these media contain additional nutrients and have an osmolarity more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO_2 will be sufficient, but for other media, a 7.5% CO_2 concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO_2 concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell prolifer-

ation. Addition of [3H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

Anticipated Results

For proliferative assays described in the basic protocol, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-Vβ), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in critical parameters. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

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Details the MLC proliferation assay.

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Exhibit 8

Dendritic cells are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

The Dendritic Cell Advantage: New Focus For Immune-Based Therapies

by Ralph M. Steinman

Summary

Dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. The best-studied function of DCs is to convert antigens into immunogens for T cells. The "DC advantage" entails a myriad of functions. DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity. Another special feature of DCs is their location and movement in the body; DCs are stationed at surfaces where antigens gain access to the body. The events that make up the life history of DCs are now being unraveled in molecular terms. As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent. © 2000 Prous Science. All rights reserved.

The focus of immune therapeutics has been on lymphocytes, the cellular mediators of immunity, and the suppression of lymphocyte function. The drug ciclosporin (cyclosporine) is an excellent and successful example. However, medicine needs therapies that enhance immunity or resistance to infections and tumors. Medicine also needs strategies, whether suppressive or enhancing, that are specific to the disease-causing stimulus or antigen. In contrast to lymphocytes, dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. DCs capture antigens and then initiate and control the activities of lymphocytes, including the development of resistance to infections and tumors (reviewed in references 1–3).

The controlling role of DCs is best known for thymus-dependent lymphocytes or T cells which are important in many diseases, the most poignant being the AIDS epidemic (Table 1). DCs were identified in a few laboratories that were focusing on the induction of immunity from resting T cells. It was noted that immune tissues (spleen, lymph nodes, lymph, blood) had a small fraction of cells with unusual "tree-like" or "dendritic" processes. These distinctive cells had not been recognized previously and they proved to have distinct functions. Most importantly, DCs were potent inducers of immunity even in animals, not just the test tube, and now even in patients (reviewed in references 1–3).

The DC field was held back by the fact that there were so few cells relative

TABLE I: HUMAN DISEASES THAT INVOLVE T CELLS

- Rejection of organ transplants and graft-vs-host disease in bone marrow transplantation
- Resistance to many infections including vaccine design
- Vaccines against tumors and immune therapies for existing tumors
- Allergy
- AIDS
- Autoimmune diseases like insulin-dependent (juvenile) diabetes, multiple sclerosis, rheumatoid arthritis and psoriasis
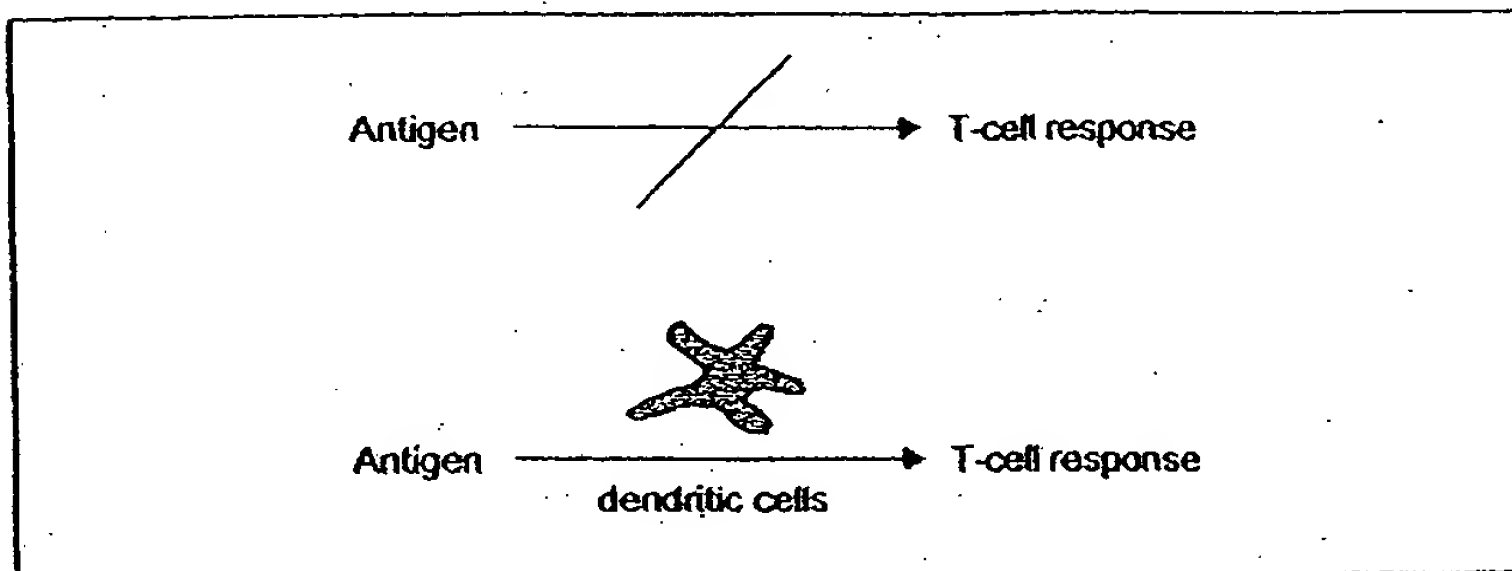

Fig. 1. A key function of dendritic cells. Antigens within tumors, transplants and infectious agents need to be presented by DCs to become immunogens, i.e., to make T cells begin to grow and exhibit their helper and killer functions.

to other players in the immune system such as B cells, T cells and macrophages. In reality, DCs are quite abundant for the job they have to do, namely, to initiate immune responses from antigen-specific T cells. In immune system organs like lymph nodes, DCs form an extensive network throughout the T cell-rich regions and physically outnumber any given antigen-reactive T cell by at least 100 to 1. The DC field was also held back because many thought that the cells were no different from macrophages, thus keeping investigators from working on the active DCs. In reality, DCs were identified on the basis of profound differences from macrophages, and their many distinct properties and functions were only uncovered by separating DCs from macrophages.

The best-studied function of DCs is to convert antigens into immunogens for T cells. The antigen receptors on T cells do not focus on intact proteins in microbes and tumors, but instead recognize fragmented or processed proteins, that is, peptides. The processing of protein antigens into peptides occurs within cells, and then the peptides are displayed or presented at the cell surface affixed to products of the major histocompatibility complex (MHC). The ensuing interaction between a T-cell receptor (TCR) and its specific MHC-peptide complex allows a T cell to detect peptides formed within cells in transplants, tumors, sites of infection and self tissues attacked during autoimmune disease (Table I). "Antigens" refers to specific substances recognized by the immune system, while "immunogens" refers to antigens that effectively induce responses either by themselves or together with enhancing materials called "adjuvants." For T cells in particular, antigens and immunogens are not one and the same (Fig. 1). Even preprocessed peptides and MHC-peptide complexes are weak immunogens. This was evident early on in the work of Peter Medawar, the great scientist who discovered the immune basis of transplantation. He spent many years trying to purify functioning transplantation antigens. These efforts were to little avail.

What was not known in Medawar's time is that transplantation antigens (later shown to be MHC-peptide complexes) become immunogenic when presented by DCs.[4] In other words, transplantation antigens when presented on many cell types are weak immunogens, but on DCs they become powerful inducers of immunity.[4] The same is true of peptides that become much more immunogenic when presented on DCs. DCs activate T cells by getting them to divide and express their helper and killer functions. Then the activated T cells interact with other antigen-presenting cells to eliminate the antigen in question. DCs are also called "nature's adjuvant," because prior adjuvants were artificial substances used to enhance immunity. The DC advantage entails a myriad of functions, some of which will be considered below.

Potency of dendritic cells in initiating immunity in tissue culture

What are some specific features of DCs that warrant attention? The first is their potency. Very small numbers of DCs are sufficient to trigger strong T-cell responses in test tubes. Immune assays are generally carried out with impure antigen-presenting cells, applied at a dose of one presenting cell for every T cell, the latter often preactivated. In contrast, roughly one DC per 30–100 T cells is more than sufficient to induce optimal responses, including responses by resting T cells. A single DC can simultaneously activate 10–20 T cells nestled within its sheet-like processes. Therefore, DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity.

It has always been clear that the accessory function of DCs did not depend exclusively on their capacity to process antigens to form MHC-peptide complexes. This is because the stimuli that were used to define the potency and immune-activating role of DCs did not require that the DCs process an applied antigen. Such stimuli included major transplantation antigens, mitogens, contact allergens, anti-

T-cell antibodies and superantigens. Furthermore, once resting T cells were activated by DCs, the T cells responded vigorously to antigens presented by other cell types, showing that the latter were not deficient in forming ligands for the antigen receptor on T cells, but instead lacked accessory properties.

The word "accessory" has since been replaced by the terms "professional" and "co-stimulatory," but the basic concept is unchanged by shifting terminology. T cells need stimuli other than their specific trigger or ligand (MHC–peptide complexes) to begin to grow and function, for example, to produce the interleukins and killer molecules mentioned above. DCs are potent in providing the needed accessory or co-stimulatory functions. For example, DCs produce an adhesion molecule called DC-SIGN that binds to a target on resting T cells called ICAM-3,[5] and DCs express very high levels of a stimulatory molecule called CD86 that binds to CD28 on resting T cells.[6] These are but two examples of the specialized activities of DCs. These cells do not operate as a single magic bullet.

Position of dendritic cells in vivo

Another special feature of DCs is their location and movement in the body. As criteria were developed to identify DCs, it became feasible to go back into the animal and patient to look for the corresponding cells in different tissues. DCs are stationed at surfaces where antigens gain access to the body (Fig. 2, left). The skin and the airway have been the best studied. DCs are found in afferent lymphatic vessels, special channels that allow cells to move from peripheral tissues to lymphoid organs, primarily the T-cell areas (Fig. 2, middle and right). This migration is most readily observed in models of skin transplantation and contact allergy, which are the two most powerful immune responses known.

DC migration is likely to be very important. The body's pool of T cells primarily traffics through the T-cell areas of lymph nodes, rather than
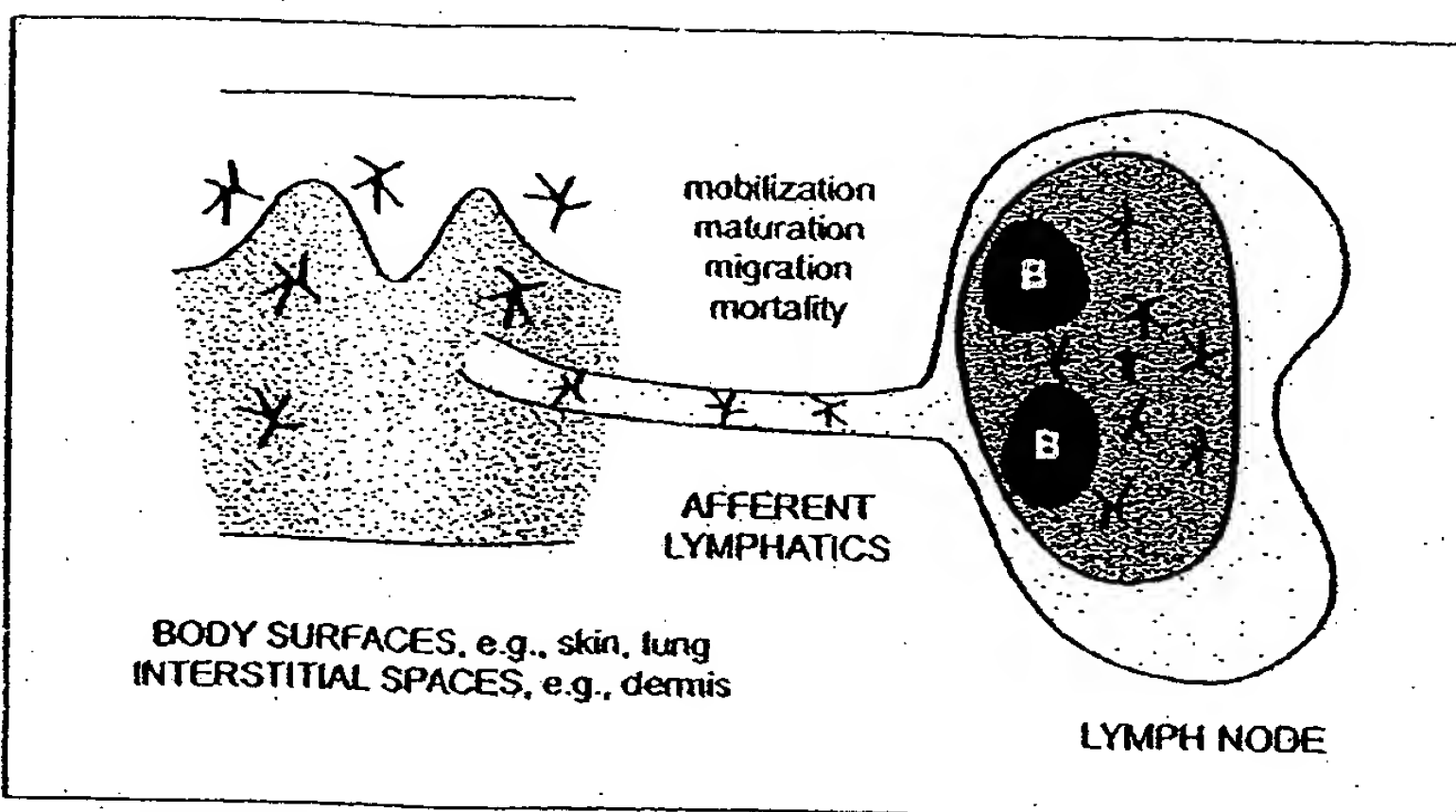
Fig. 2. Distribution of dendritic cells in situ. DCs at body surfaces and in solid organs can pick up antigens, move to the lymphoid tissues to find antigen-specific T cells and initiate immunity. Molecular mechanisms are being uncovered that govern the mobilization, maturation, migration and mortality of these DCs. In the lymph node, T lymphocytes are selected for expansion and differentiation into helper and killer T cells. The activated T cells then leave the lymph node to return to the body surface or peripheral organ to eliminate the antigen.

Labels in figure: mobilization / maturation / migration / mortality; AFFERENT LYMPHATICS; BODY SURFACES, e.g., skin, lung INTERSTITIAL SPACES, e.g., dermis; LYMPH NODE

through tissues where antigens are usually deposited. So when DCs capture antigens in the skin, airway or another peripheral tissue, their migration to the T-cell areas gives them a chance to select the corresponding rare specific T cells from the assembled repertoire (Fig. 2). The selected T cells then increase in numbers (clonal expansion) and function, enabling the specific immune response to begin. The initial frequency of T cells that recognize an antigen is very small. Only one in 10,000–100,000 of T cells in the repertoire responds to a specific MHC–peptide complex. Therefore, it is so precise and efficient for DCs to be able to pick up an antigen in the periphery and then initiate the immune response from rare T-cell clones in lymphoid organs.

The events that make up the life history of DCs (Figs. 2 and 3) are now being unraveled in molecular terms. For example, scientists are figuring out how to expand antigen-capturing precursors to DCs using flt3 ligand and granulocyte colony-stimulating factor (G-CSF). Key players for the mobilization of DCs from the periphery to lymph nodes are the multidrug resistance receptors, usually studied for their capacity to mediate resistance to chemotherapeutic agents rather than

movement of DCs. Migration of DCs is controlled by chemokines produced in the lymphatic vessels and lymphoid organs (Fig. 2). These act on DC chemokine receptors to orchestrate their movement to the T-cell areas. Then within the lymphoid tissue, several members of the tumor necrosis factor (TNF) and TNF-receptor families, such as TRANCE and CD40 ligand, trigger DC production of cytokines like interleukin-12. The TNF family also maintains DC viability. Otherwise the cells die within a day or two. Each of these components of DC function provides targets for manipulating immunity.

Priming of T-cell immunity via dendritic cells

Animal studies

During the early research on DCs, several labs administered antigens to experimental animals and then tried to identify the cells that had captured the antigens in a form that was immunogenic. Regardless of the route of antigen administration (blood, muscle, skin, intestine and airway), DCs were the major reservoir of immunogen.
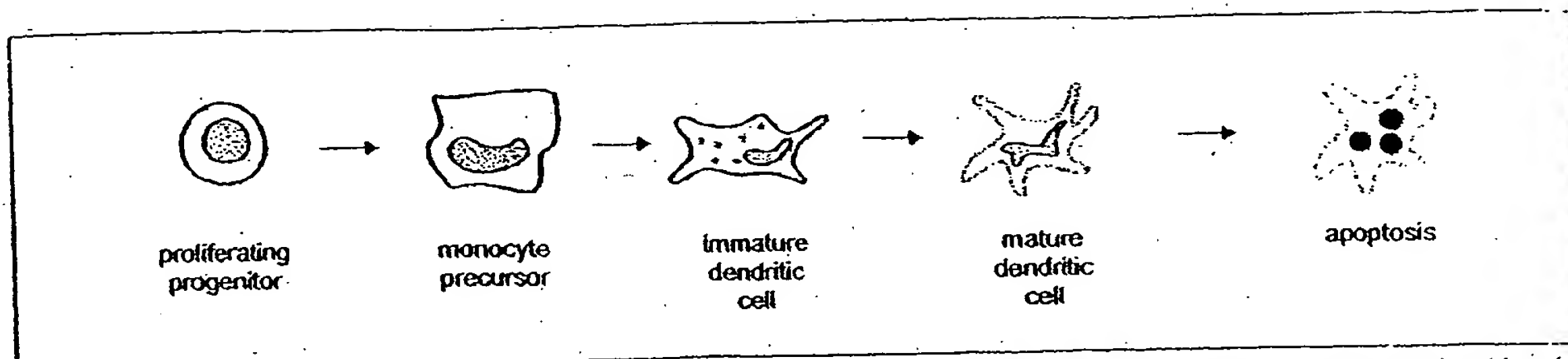
proliferating progenitor → monocyte precursor → immature dendritic cell → mature dendritic cell → apoptosis

Fig. 3. The life history of dendritic cells. DCs arise from proliferating progenitors, primarily in the bone marrow, and this can be driven by cytokines like flt-3 ligand and G-CSF. Precursors are formed, such as the monocytes in blood, which then give rise to immature DCs. The immature DCs are capable of producing large amounts of antigen-presenting MHC products and capturing antigens. Multidrug resistance receptors are newly recognized players in the mobilization of immature DCs. DCs mature in response to various stimuli such as infection and inflammation, and migrate under the influence of chemokines to the lymphoid tissues. There the DCs die within a day unless their life-span is prolonged by TNF-family molecules expressed by the activated T cells.

Next, DCs were used as nature's adjuvant to immunize animals. The DCs were taken from mice or rats, exposed to antigens *ex vivo* and injected back into immunologically naive recipients. The animals became immunized to the antigens that had been captured by the DCs, and the immunization took place in the lymph nodes draining the site of DC injection. Genetic proof was provided that the DCs were priming the animal directly and not simply handing off their antigen to other cells.[7,8]

DC-based immunization is really very different from all prior attempts at cell therapy. Immunology has had extensive experience with "passive immunization," whereby a recipient is given large numbers of cells that are activated prior to injection. It is hard to produce such large numbers of cells, and their lifespan, diversity and efficacy are all finite. In contrast, when relatively small numbers of antigen-charged DCs are used to induce immunity, this produces "active immunization." Now the animals (and patients, see below) can make their own diverse and longer lasting immune response to the antigen-bearing DCs.

Human studies

The above experiments made it clear that DCs, pulsed *ex vivo* with antigens, actively immunized animals and raised the exciting possibility that scientists would be able to induce resis-
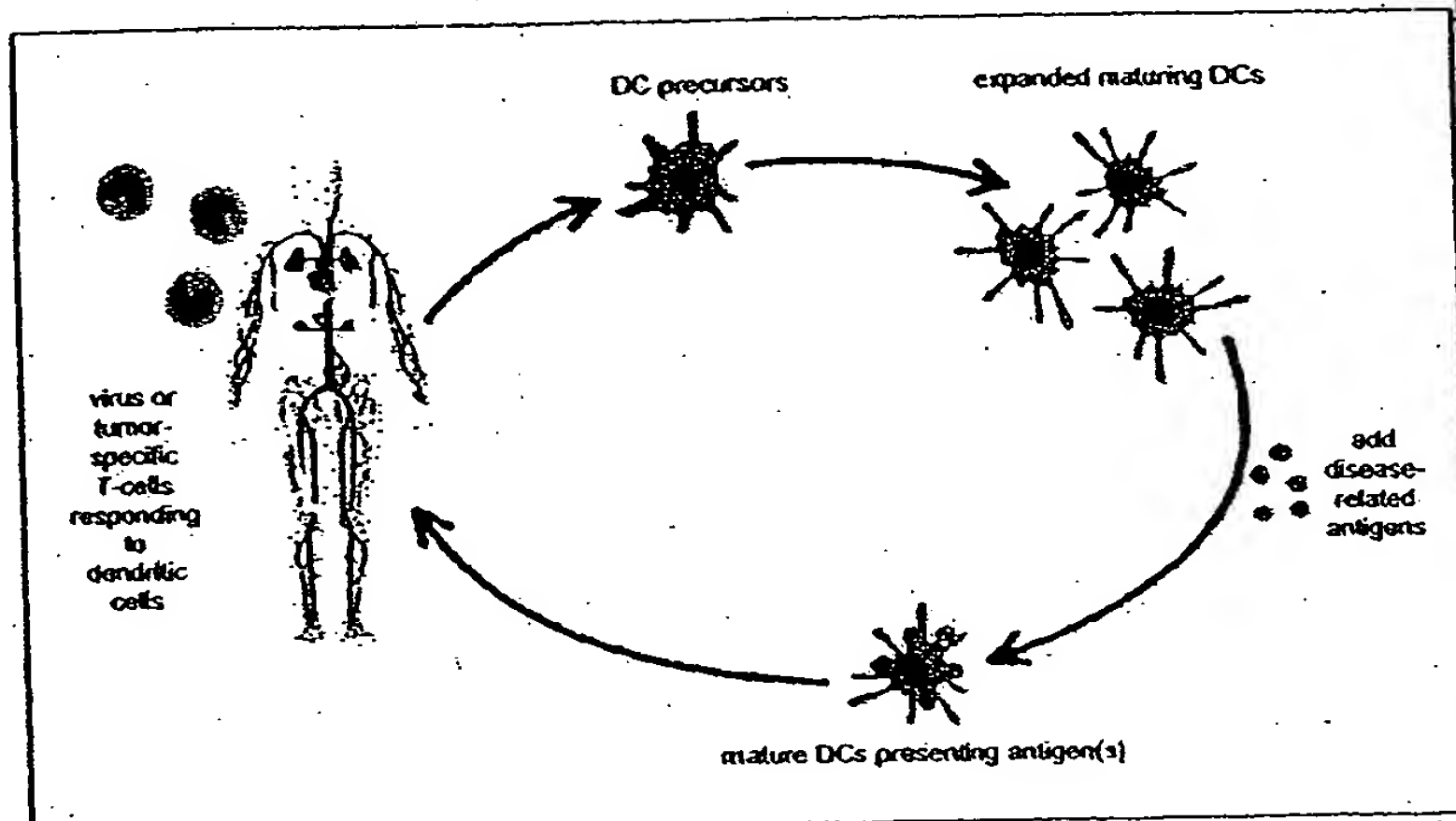
Fig. 4. The use of dendritic cells as adjuvants for enhancing immunity to tumors and infectious agents in humans. This new form of immune therapy begins with the isolation of DC precursors from the patient, usually from blood. The precursors develop *ex vivo* (in relatively simple tissue culture systems) into large numbers of more mature DCs. During this time, the DCs are charged with antigens from the tumor or infection. Then the DCs are reinfused to elicit immunity and thereby resistance to the disease.

tance to tumors, infections and transplants in patients. For example, could one expose patients' DCs *ex vivo* to antigens in their tumors and then reinfuse the antigen-bearing DCs to elicit tumor-specific immunity (Fig. 4)? This approach is actually not terribly complicated, but one first had to overcome a major obstacle and learn to generate large numbers of DCs. These techniques became available in the 1990s. They have energized the field and, accordingly, clinical trials for the immunization of humans against cancer have begun on most continents.

It is evident that DCs can serve as adjuvants for humans, converting antigens into immunogens.[9,10] Even in advanced cancer, immune responses already have been observed that are similar to or better than immune responses obtained with other approaches. However, this approach is still in its preliminary stages, since a good deal of science remains to be developed. On the one hand, there are critical unknowns in terms of overall DC biology. Many of the clinical studies to date, for example, have overlooked key features that could improve DC function, such as the need for DCs

to be sufficiently mature (see below) to be effective *in vivo*. Also, DC biology has to be placed in the context of specific tumors and pathogens and patients for DC-based therapies to be optimized.

To summarize and further illustrate the role of DCs in the context of human disease (Table I), consider the need to harness T cells to resist tumors and chronic infections. Protein antigens often are known for a tumor-like melanoma, or for a virus like HIV-1 whose genetic sequence has been available for more than 15 years. However, this knowledge about antigens from melanoma and HIV-1 antigens remains to be converted into methods that provide better immunogens either for immune therapy of melanoma or for the design of HIV-1 vaccines. This is because some important facts of immunological life are being overlooked. When antigens are injected, they also need to gain access to the right DCs to become immunogens (Fig. 1).

Delivering antigens to dendritic cells

Broadly speaking, a central goal is to learn how to deliver or "target" antigens to DCs and simultaneously to differentiate or "mature" the cells to their most potent state. These two challenges, antigen targeting and DC maturation, prove to be intertwined.

Targeting means that the antigen should be in a form that the DCs can recognize. Without such recognition, the uptake and subsequent processing of antigen to form MHC-peptide complexes is suboptimal. DCs have a number of special mechanisms for capturing antigens and converting these into MHC-peptide complexes (Table II). For example, DCs have a receptor called DEC-205 whose binding partners or ligands are still unknown. Nonetheless, it is clear that DEC-205 greatly increases the capacity of DCs to form MHC-peptide complexes.[11] DCs also carry out a fascinating process called "cross-presentation." DCs can take up dying cells and effi-

ciently extract peptides from them, so antigens "cross" from the dying cell to the DC. The discoverers of this phenomenon called it "resurrecting the dead."[12] Cross-presentation allows DCs to efficiently form MHC-peptide complexes from dead cells in tumors, transplants and tissues under autoimmune attack.

Special uptake and processing mechanisms allow DCs to tailor a protein antigen, as well as the proteins in a complex microbe or tumor cell, into peptides that bind to an individual's MHC products. The latter are exceptionally polymorphic, differing genetically from one individual to another. As a result, the relevant immunizing peptides differ from one individual to another. One reason why peptides are not ideal immunogens is that they must be individualized. DCs, in contrast, can capture antigens with high efficiency and likewise extract peptides that are relevant for any individual.

A second DC advantage is that these cells have the many required accessory or co-stimulatory properties for converting the selected peptides ("antigens") into effective immunogens. A third DC advantage is that these cells position themselves in a way that leads to the identification of rare antigen-reactive T lymphocytes *in vivo* (Fig. 2). DCs thus overcome many of the difficult obstacles in initiating immunity.

In order for an antigen to be a strong immunogen, one needs to provide a stimulus for the final differentiation or maturation of the DCs (Fig. 3). Most DCs in the body are in an immature state and lack many features that lead to a strong T-cell response.

Immature DCs, for example, lack the CD86 and CD40 molecules that greatly boost the DC-T cell interaction. Immature DCs also lack a chemokine receptor called CCR7 that seems very important for proper migration and homing to lymph nodes to start immunity. For cancer immunology, it is unlikely that tumors provide maturation stimuli. Tumors may even block DC maturation induced by other stimuli. Therefore it is important to learn how to deliver tumor cells to DCs and bypass the normal obstacles to effective antitumor immunity.

Surprising recent evidence actually links DC maturation to the efficient formation of MHC-peptide complexes or TCR ligands (Table II). Immature DCs take up antigens, but they do not make abundant MHC-peptide complexes until they receive a maturation stimulus.[13,14] Maturation also up-regulates CD86 co-stimulators, but the CD86 actually travels together with the TCR ligands to the surface of the DCs. At the DC surface, the MHC molecules and CD86 remain clustered with each other, keeping the machinery for T-cell activation juxtaposed. This phenomenon will help explain the potency of DCs, because TCR ligands and co-stimulators are displayed together on the cell surface and in high levels.

Control points beyond antigen targeting and maturation of DCs

Research on DCs is moving more vigorously, because the cells are more readily available and because their role in the immune system is considered essential. Nonetheless, researchers in this field are just beginning to find ways to manipulate DCs *in situ*. Putting together an antigen that targets

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TABLE II: DENDRITIC CELL SPECIALIZATION TO INCREASE MHC-PEPTIDE COMPLEX FORMATION

- Receptors for antigen uptake, e.g., DEC-205
- Processing of dying cells, nonreplicating microbes and immune complexes onto MHC class I ("cross-presentation")
- Regulation of antigen processing by maturation stimuli
- Clustering of T-cell receptor ligands with co-stimulators like CD86

to DCs with a stimulus for DC maturation will be a major step in improving the conversion of antigens into immunogens, as in immune-based therapies against tumors and infectious agents.

Additional challenges and questions are evident:

- How can DC numbers be increased *in situ* and how can active DCs be mobilized to a cancer or site of chronic infection?

- Can DCs induce strong immune memory to make vaccination long lasting and effective (we have only been reviewing the role of DCs in the initiation of immunity)?

- Can DCs change the quality of the immune response? "Quality" refers to recent evidence for different types of DCs, especially a subset that induces Th1-type T cells for resistance to infectious agents and strong memory.

- Is it possible to move beyond DC-based immunization experiments and use DCs to either regulate or tolerize the immune system, as frequently required in transplantation and autoimmune diseases?

- Can DCs influence elements of the immune system other than T cells; for example, B cells and the innate defenses provided by natural killer (NK) and NK-T cells?

The answer to all these questions is a preliminary "yes." As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

Dendritic cells and better control of disease

DCs provide important avenues for the investigation of human disease. Many labs are exploiting DCs to identify antigens relevant for immunity against human pathogens. In these experiments, one introduces complex but clinically important antigens to DCs and then identifies which components are best presented to the immune system. We have recently used this approach to identify previously un-

known immune responses to the Epstein-Barr virus,[15] a virus we all carry that has the potential to cause cancer like Hodgkin's lymphoma. Other laboratories have been using DCs to identify new antigens in other infectious agents, in transplants and in cancers like melanoma.

Investigators are also manipulating DCs *ex vivo* and then reinfusing the cells to identify conditions leading to strong immunity in patients (Fig. 4). In particular, DC-mediated active immunization against cancer is being vigorously pursued, as mentioned above. Instead of manipulating DCs *ex vivo*, a more desirable goal would be able to alter DCs directly *in situ*. Some approaches are under way. An example is the injection of cytokines like flt3 ligand and G-CSF to mobilize various precursor populations of DCs. One should also develop methods to control DC mobilization, migration and maturation. In sum, DCs are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

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Eth C

Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor

(heterodimeric lymphokine/T-cell growth factor/lymphokine-activated killer cells/coordinate gene regulation/interleukin-12)

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ABSTRACT   Cytotoxic lymphocyte maturation factor (CLMF) is a disulfide-bonded heterodimeric lymphokine that (i) acts as a growth factor for activated T cells independent of interleukin 2 and (ii) synergizes with suboptimal concentrations of interleukin 2 to induce lymphokine-activated killer cells. We now report the cloning and expression of both human CLMF subunit cDNAs from a lymphoblastoid B-cell line, NC-37. The two subunits represent two distinct and unrelated gene products whose mRNAs are coordinately induced upon activation of NC-37 cells. Coexpression of the two subunit cDNAs in COS cells is necessary for the secretion of biologically active CLMF; COS cells transfected with either subunit cDNA alone do not secrete bioactive CLMF. Recombinant CLMF expressed in mammalian cells displays biologic activities essentially identical to natural CLMF, and its activities can be neutralized by monoclonal antibodies prepared against natural CLMF. Since this heterodimeric protein displays the properties of an interleukin, we propose that CLMF be given the designation interleukin 12.

The molecular cloning and expression of recombinant cytokines has made possible both significant advances in our understanding of the molecular basis of immune responses and the development of new approaches to the treatment of disease states. As an example, recombinant interleukin 2 (recombinant IL-2) has been shown to be capable of causing regression of established tumors in both experimental animals (1) and in man (2); however, its clinical use has been associated with significant toxicity (2). One potential approach to improving the therapeutic utility of recombinant cytokines is to use them in combination (3, 4). With this concept in mind, we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes in vitro and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 in vivo. This led to the identification of a factor, designated cytotoxic lymphocyte maturation factor (CLMF), that synergized with recombinant IL-2 to facilitate the generation of both cytolytic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells in vitro (5, 6). CLMF was subsequently purified to homogeneity from a human lymphoblastoid B-cell line (NC-37) and was shown to be a 75-kDa disulfide-bonded heterodimer composed of two subunits with molecular masses of 40 kDa and 35 kDa (7).¶ We now report the molecular cloning and expression of CLMF.

MATERIALS AND METHODS

cDNA Cloning. A subline of NC-37 cells selected for its ability to produce high levels of CLMF (7), NC-37.98, was induced with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 for 16 hr. Poly(A)⁺ RNA was isolated, and random hexamer-primed cDNA libraries were established in phage λgt10 by standard procedures. Mixed-primer polymerase chain reaction (PCR) using controlled ramp times (8) was performed as follows. PCR primers contained all possible codons and were 14 or 15 nucleotides long (Fig. 1) with a 5′ extension of 9 nucleotides containing an EcoRI site for subcloning. Degeneracies varied from 1 in 32 to 1 in 4096; 0.5–4 pmol per permutation of forward and reverse primer was used in a 50- to 100-μl PCR mixture with 40 ng of cDNA made from NC-37.98 cells that had been activated by culture with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml for 16 hr (40-kDa subunit) or with 3 μg of human genomic DNA (35-kDa subunit). PCR cycling parameters were as follows. Initial denaturation was at 95°C for 7 min. Low-stringency annealing was performed by cooling to 37°C over 2 min, incubating 2 min at 37°C, heating to 72°C over 2.5 min, extending at 72°C for 1.5 min, heating to 95°C over 1 min, and denaturing at 95°C for 1 min. This cycle was repeated once. Thirty standard cycles (40-kDa subunit) or 40 standard cycles (35-kDa subunit) were performed as follows: 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. "Amplicons" of the expected size were gel-purified, subcloned, and sequenced. The 40-kDa subunit cDNAs were isolated by hybridizing the 54-mer amplicon in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 20% formamide at 37°C overnight. Filters were washed in 2× SSC at 42°C for 30 min and exposed to x-ray film. The 35-kDa subunit cDNAs were isolated by hybridizing the 51-mer amplicon in 5× SSC/20% formamide at 37°C overnight. The filters were washed in 2× SSC at 40°C for 30 min and exposed to x-ray film. Positive clones were plaque-purified, their inserts were subcloned into the pBluescript plasmid, and their sequences were determined by using Sequenase.

Expression. cDNAs were separately engineered for expression in vectors containing the simian virus 40 early promotor essentially as described (9). COS cells were transfected with both CLMF subunit expression plasmids mixed together or

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Abbreviations: CLMF, cytotoxic lymphocyte maturation factor; rCLMF and nCLMF, recombinant and natural CLMFs; CTL, cytolytic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; n, natural; PCR, polymerase chain reaction.
†To whom reprint requests should be addressed.
¶The cDNA sequences reported in this paper have been deposited in the Genbank data base [accession nos. M38443 (35-kDa CLMF subunit) and M38444 (40-kDa CLMF subunit)].

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                                          ---->          <----
  1   MCPARSLLLV ATLVLLDHLS LARNLPVATP DPGMFPCLHH SQNLLRAVSN

 51   MLQKARQTLE FYPCTSEEID HEDITKDKTS TVEACLPLEL TKNESCLNSR

101   ETSFITNGSC LASRKTSFMM ALCLSSIYED LKMYQVEFKT MNAKLLMDPK

151   RQIFLDQNML AVIDELMQAL NFNSETVPQK SSLEEPDFYK TKIKLCILLH

201   AFRIRAVTID RVTSYLNAS
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                                          ---->          <-----
  1   MCHQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVVLTC

 51   DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS

101   LLLLHKKEDG IWSTDILKDQ KEPKNKTFLR CEAKNYSGRF TCWWLTTIST

151   DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP

201   AAEESLPIEV MVDAVHKLKY ENYTSSFFIR DIIKPDPPKN LQLKPLKNSR

251   QVEVSWEYPD TWSTPHSYFS LTFCVQVQGK SKREKKDRVF TDKTSATVIC

301   RKNASISVRA QDRYYSSSWS EWASVPCS
```

Fig. 1. Amino acid sequences of the 35-kDa (*Upper*) and 40-kDa (*Lower*) CLMF subunits as deduced from the respective cDNAs and shown in single-letter code. Signal peptides are overlined, cysteine residues are marked by a caret, and N-linked glycosylation sites (NXS, NXT, where X is another amino acid) are underlined. The peptide sequences used to generate PCR probes are overlined with arrows indicating the direction of amplification.

with each one separately by the DEAE-dextran method. Twenty-four hours after transfection, the serum-containing medium was replaced with medium containing 1% Nutridoma-SP (Boehringer Mannheim), and supernatant fluids were collected from the cultures after 40 hr. These fluids were stored at 4°C until tested in the bioassays.

General Methods. Standard molecular biological procedures were used as described (10). CLMF bioassays were performed as detailed (7).

Computer Searches. The National Biomedical Research Foundation protein data base (Release 26.0) as well as the Genbank and European Molecular Biology Laboratory databases (Releases 65.0 and 24.0, respectively) were searched for sequences homologous to CLMF cDNAs. The two subunit sequences were compared to each other using the ALIGN program (mutation data matrix, break penalty of 6; see ref. 11).

RESULTS

Partial N-terminal amino acid sequences of the two CLMF subunits (7) were used to generate completely defined 51- to 54-base-pair (bp)-long oligonucleotide probes by means of mixed primer PCR. These probes were used to screen cDNA libraries made from RNA from activated NC-37.98 cells, and cDNAs encoding the two subunits were isolated and characterized. Both cDNAs encode secreted proteins with a classical hydrophobic N-terminal signal peptide immediately followed by the N terminus of the mature protein as determined by protein sequencing (7). Two independent cDNA clones for the 40-kDa subunit were shown to be identical. Both encode the mature 40-kDa subunit that is composed of 306 amino acids (calculated $M_r = 34,699$) and contains 10 cysteine residues and four potential N-linked glycosylation sites (Fig. 1). Two of these sites are within isolated tryptic peptides derived from the purified 40-kDa CLMF subunit protein. Amino acid sequence analysis has shown that Asn-

222 is glycosylated, whereas Asn-125 is not (Fig. 1; F. Podlaski, personal communication). The mature 35-kDa subunit is composed of 197 amino acids (calculated $M_r = 22,513$), with 7 cysteine residues and three potential N-linked glycosylation sites (Fig. 1). When purified CLMF is reduced with 2-mercaptoethanol and analyzed by SDS/PAGE, the 35-kDa subunit appears to be heterogeneous, suggesting that it may be heavily glycosylated (7). Two variants of 35-kDa subunit-encoding cDNAs were isolated. The first type had the sequence shown in Fig. 1. Additional isolates contained what is probably an allelic variation, replacing Thr-213 with a methionine residue.

Computer searches of sequence databases showed that the amino acid sequences of the two subunits are not related to any known protein. The subunit sequences are also not related to each other, since a comparison using the ALIGN program (11) gave a score of 1.27; only scores >3 are considered to indicate significant evolutionary relationship (12). The genes encoding the subunits appear to be unique, since low- and high-stringency hybridizations of genomic blots revealed identical banding patterns (data not shown). RNA blots showed the size of the 40-kDa subunit mRNA to be 2.4 kb, whereas the 35-kDa subunit was encoded by a 1.4-kb transcript (Fig. 2). Expression of the two mRNAs encoding the subunits was coordinately regulated upon induction (Fig. 2). When NC-37.98 cells were activated with PMA and calcium ionophore for 72 hr, mRNA encoding each of the CLMF subunits was minimally detectable at 6 hr after the beginning of induction but was readily detected at 24 hr and continued to accumulate until maximal levels were reached at 72 hr (normalized to GAPDH mRNA levels; see the legend to Fig. 2). In contrast, the mRNA for IL-2 in activated NC-37.98 cells was already at high levels at 6 hr and subsequently decreased, whereas the mRNAs for the low-affinity IL-2 receptor (p55) followed the induction pattern seen for the CLMF subunits. Scanning of RNA blots also revealed that steady-state mRNA levels for the 40-kDa

Immunology: Gubler et al.
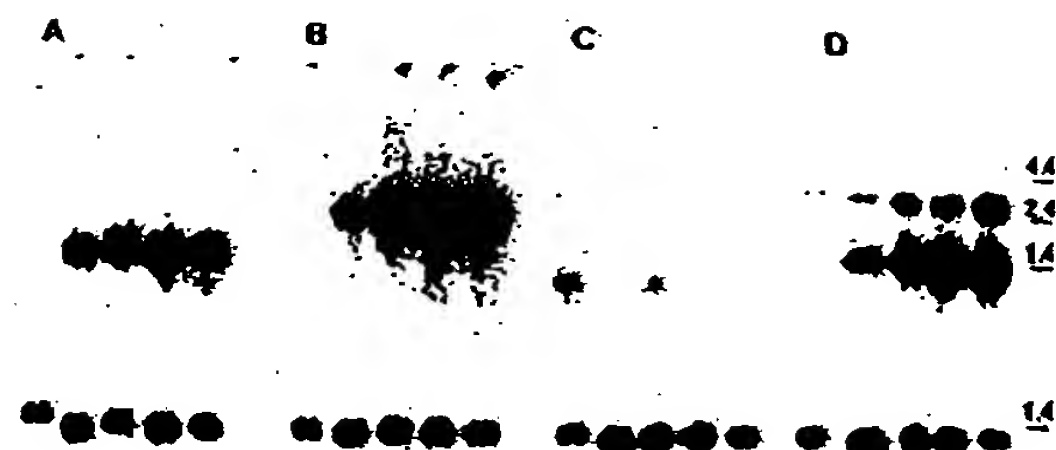
Proc. Natl. Acad. Sci. USA 88 (1991)   4145

Fig. 2. RNA blots showing the coordinate induction of the 35-kDa (A) and 40-kDa (B) CLMF subunit mRNAs and IL-2 mRNA (C) and its p55 receptor mRNA (D). Poly(A)⁺ RNA (5 μg) from NC-37.98 cells activated with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml were loaded in each lane. Lanes from left to right in each panel show RNAs isolated 6, 24, 30, 48, and 72 hr after induction, respectively. (Upper) Four-day exposures. (Lower) Two-hour exposure of the same blots after stripping and rehybridization with a GAPDH probe. Marker sizes are in kb (BRL RNA ladder).

CLMF subunit were severalfold higher than those for the 35-kDa subunit expressed by the same cells. This finding parallels the observation that activated NC-37 cells secrete excess free 40-kDa subunit protein (7). The 3' untranslated sequences of both CLMF subunit mRNAs contain several copies of the octamer motif TTATTTAT (data not shown). This sequence is present in other transiently expressed mRNAs and is involved in regulating mRNA stability (13).

Coexpression of the 40-kDa and 35-kDa CLMF subunit cDNAs in COS cells was required to generate secreted biologically active CLMF (Table 1 and Fig. 3). COS cells transfected with cDNA encoding either the 40-kDa subunit alone or the 35-kDa subunit alone did not secrete biologically active CLMF (Table 1). Mixing media conditioned by COS

cells that had been separately transfected with one or the other of the two CLMF subunit cDNAs also did not give rise to bioactive CLMF (Table 1).

Two types of assays were used to compare rCLMF and nCLMF. The first assay measures the proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes, whereas the second assay evaluates the synergy between CLMF and suboptimal concentrations of IL-2 in the generation of LAK cells in hydrocortisone-containing cultures (7). The data in Fig. 3 show that rCLMF as expressed in COS cells and nCLMF as purified from NC-37 cells are essentially identical. Dose-response curves for rCLMF and nCLMF were superimposable in each of the two assays, and rCLMF was neutralized by a monoclonal antibody raised against nCLMF. Conditioned media from cultures of mock-transfected COS cells displayed no activity in these assays (Table 1 and data not shown).

DISCUSSION

In a previous report (7), we described the purification of a heterodimeric cytokine, CLMF, that synergized with low amounts of IL-2 to cause the generation of LAK cells in the presence of hydrocortisone and stimulated the proliferation of activated T cells independent of IL-2. In the present report, we have used the N-terminal amino acid sequence information previously obtained to clone the two subunit cDNAs of CLMF. Protein purification of NC-37 cell line-derived CLMF had shown that the protein was composed of two disulfide-bonded subunits with different N-terminal amino acid sequences (7). However, it was not clear from our previous results whether the two subunits were processed from one common gene product and whether proteolytic posttranslational processing other than signal peptide cleavage was occurring. The molecular cloning and sequencing of

Table 1. Coexpression of both CLMF subunit cDNAs is required for secretion of biologically active CLMF by COS cells

Addition	Conc., units/ml	Dilution	[³H]Thymidine incorporated by PHA-activated lymphoblasts, mean cpm ± 1 SEM
Cytokine*			
None	—		11,744 ± 514
nCLMF	200		68,848 ± 878
nCLMF	40		48,827 ± 605
nCLMF	8		26,828 ± 594
nCLMF	1.6		17,941 ± 196
Culture fluid from COS cells transfected with			
A. 35-kDa CLMF subunit cDNA		1:20	11,912 ± 660
		1:100	10,876 ± 232
B. 40-kDa CLMF subunit cDNA		1:20	11,699 ± 931
		1:100	11,666 ± 469
C. 35-kDa + 40-kDa CLMF subunit cDNAs		1:20	58,615 ± 587
		1:100	38,361 ± 828
1:1 mix of culture fluids A and B		1:10†	11,544 ± 483
		1:50	10,503 ± 259
CM from mock-transfected control‡		1:20	11,503 ± 286
		1:100	10,751 ± 303

PHA-activated lymphoblasts were prepared from human peripheral blood mononuclear cells as described (7). Lymphoblast proliferation was measured in a 48-hr assay (7) in which 2 × 10⁴ lymphoblasts were incubated in 100-μl cultures containing the indicated amounts of natural CLMF (nCLMF) or COS cell culture fluids. [³H]Thymidine was added to each culture 18 hr prior to harvest. Conc., concentration.
*nCLMF is purified NC-37-derived CLMF.
†1:10 dilution of the 1:1 mixture of culture fluids A and B was equivalent to a 1:20 final dilution of each of the individual culture fluids.
‡Conditioned medium (CM) from cultures of mock transfected COS cells.
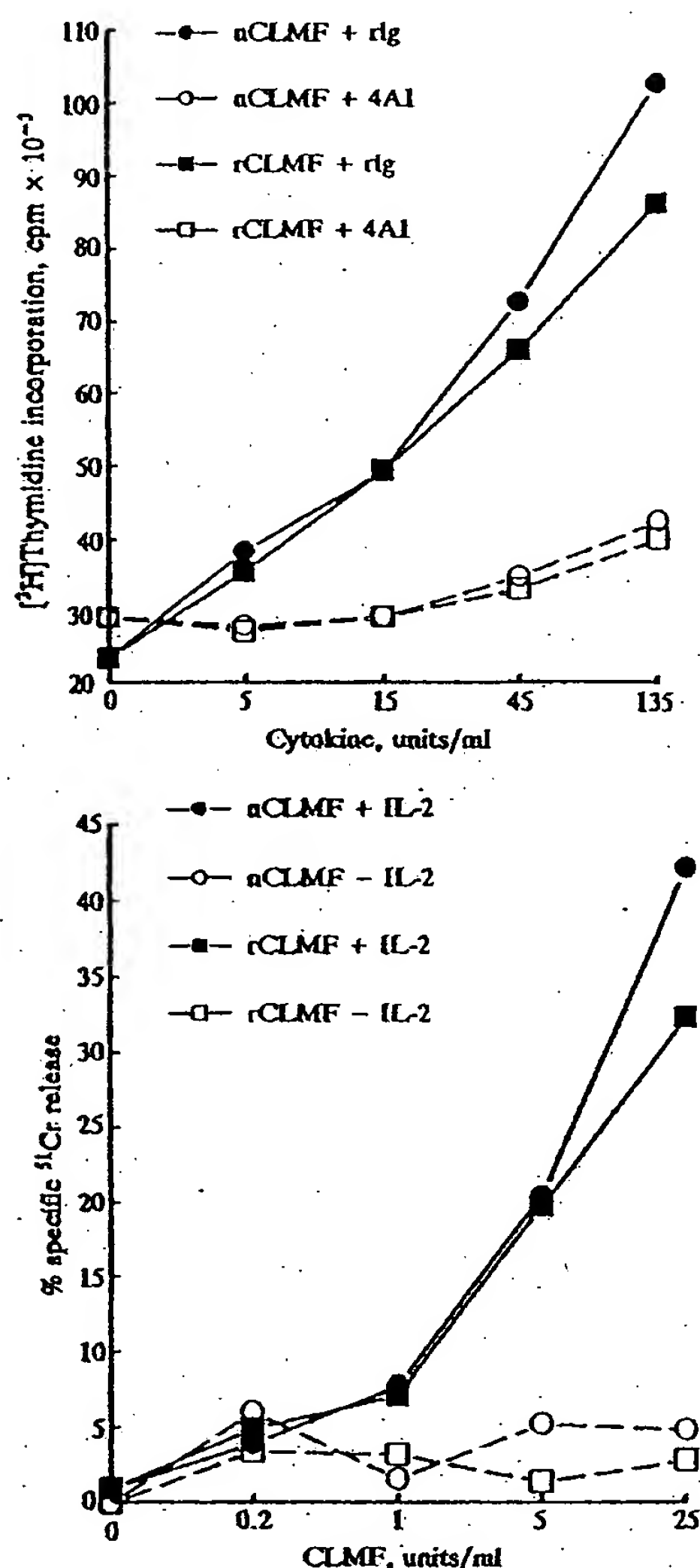
FIG. 3. Comparison of biologic activities of nCLMF (circles) and recombinant CLMF (rCLMF, squares). nCLMF was purified from NC-37 cell-conditioned media; rCLMF was purified from conditioned media from cultures of COS cells transfected with cDNAs encoding the 40-kDa and 35-kDa human CLMF subunits. (*Upper*) T-cell growth factor assay. The ability of CLMF to stimulate the proliferation of human PHA-activated lymphoblasts in 48-hr cultures was assayed as described (7). CLMF preparations were mixed with neutralizing rat monoclonal anti-human CLMF antibody 4A1 (ref. 7; open symbols) or with normal rat IgG (Sigma; rIg, closed symbols) at a final concentration of 20 μg of IgG/ml and were incubated for 30 min at 37°C prior to addition of PHA blasts. All values are means of triplicate determinations. (*Lower*) LAK cell induction assay. The ability of CLMF, alone or in combination with recombinant IL-2, to induce the generation of LAK cells in 4-day cultures was assessed as described (7). Low-density peripheral blood lymphocytes were incubated in the presence of various amounts of nCLMF or rCLMF with (closed symbols) or without (open symbols) recombinant IL-2 at 7.5 units/ml. Units of CLMF activity were based on previous titrations in the T-cell growth factor assay. Hydrocortisone sodium succinate (Sigma) was included at a concentration of 0.1 mM to minimize triggering of endogenous cytokine cascades. Lysis of [51]Cr-labeled Daudi cells was assessed at an effector/target ratio of 5:1. The data shown represent the means of quadruplicate determinations. The spontaneous [51]Cr release was 20%.

the corresponding cDNAs now has demonstrated that there is no common precursor for the two CLMF subunits; rather, they are encoded by completely different genes. The predicted and actual amino acid composition for the two subunits are strikingly similar; differences in predicted versus actual molecular weights are accounted for by glycosylation (F. Podlaski, personal communication). Thus, the only major posttranslational proteolytic event that appears to take place in the maturation of the CLMF subunits is signal peptide cleavage.

The kinetics of expression of the individual CLMF subunit mRNAs were examined and compared to the expression of mRNAs for IL-2 and the IL-2 receptor p55. Previously it had been observed that NC-37 cells, like certain murine (14) and marmoset (15) B-cell lines, secreted IL-2 when activated (M.K.G., unpublished results). RNA blots demonstrated that upon activation of NC-37 cells, both CLMF subunit mRNAs were coordinately induced with kinetics similar to the IL-2 receptor (p55) mRNAs. On the other hand, IL-2 mRNA levels peaked much earlier. Similar differences in induction kinetics were also seen at the level of IL-2 and CLMF bioactivity secreted from NC-37 cells (M.K.G., unpublished data). These kinetic differences are consistent with our previous observation that in a cytolytic lymphocyte response, IL-2 appears to act earlier than CLMF (5).

Transfection studies with COS cells established that only coexpression of both subunit cDNAs gives rise to secreted bioactive CLMF. Thus, it appears that the two proteins have to interact within the endoplasmic reticulum to assemble properly into bioactive secreted CLMF. By comparing the activity of rCLMF to that of nCLMF in the T-cell growth factor and LAK cell induction assays (Fig. 3) and assuming that the specific activity of rCLMF is similar to that of nCLMF [8 × 10⁷ units/mg (7)], we estimate that the amount of rCLMF heterodimer produced in these experiments was 5–50 ng/ml. The finding that COS cells, which are fibroblast-like cells, are able to assemble correctly the two CLMF subunits to form bioactive CLMF indicates that this secretion and processing pattern is not limited to cells of the lymphoid lineage.

Western blot analysis using an anti-CLMF antibody specific for the 40-kDa subunit has allowed confirmation that (*i*) COS cells transfected with both CLMF subunit cDNAs secrete CLMF with the expected heterodimeric structure and (*ii*) COS cells transfected with the 40-kDa subunit cDNA alone secrete that subunit (F. Podlaski, personal communication). Since no bioactivity was detected in media conditioned by COS cells transfected with only the 40-kDa subunit, that subunit by itself appears either to have a much reduced specific activity compared with heterodimeric CLMF or to be completely inactive.

Because of the lack of a high-affinity antibody specific for the 35-kDa subunit, we have not yet been able to determine definitively whether COS cells transfected with only the 35-kDa subunit cDNA secrete that subunit. Since no bioactivity was detected in the media, secretion of a bioactive 35-kDa subunit by itself could be very inefficient; alternatively, similar to the 40-kDa subunit, the protein could be much less active or inactive altogether. Intracellular 35-kDa protein in the absence of the other subunit could be inherently unstable; there is precedence for this phenomenon, since it has been reported that 90% of the β chains of lutropin (LH), when expressed in the absence of α chains, are retained in the endoplasmic reticulum and are slowly degraded (16). Simple mixing of media conditioned by COS cells transfected separately with either one of the two CLMF subunit cDNAs did not yield bioactive CLMF. One possible explanation would be that the cells do not secrete the 35-kDa subunit by itself. More likely, our experimental conditions did not allow proper heterodimer formation. One would expect that only

carefully controlled renaturation and oxidation conditions would allow the disulfide bond formation required for generation of bioactive CLMF.

Normal human peripheral blood lymphocytes under the appropriate induction conditions produce both CLMF subunit mRNAs and secrete the active protein (N.N. and M.K.G., unpublished data). There is some evidence suggesting that CLMF is produced predominantly by B cells. In preliminary experiments, B-cell mitogens have appeared to be more effective than T-cell mitogens in eliciting CLMF production from peripheral blood lymphocytes (M.K.G., unpublished results). When screening human cell lines for their ability to produce CLMF activity (7), we observed that four of eight B-cell lines tested produced CLMF after activation with PMA and calcium ionophore, whereas none of five T-cell lines produced CLMF. Nevertheless, three of these T-cell lines secreted large amounts of IL-2 and tumor necrosis factor activity after activation (M.K.G., unpublished results). Likewise, natural killer cell stimulatory factor (NKSF), a heterodimeric cytokine similar or identical to CLMF, was isolated from RPMI 8866 lymphoblastoid B cells (17). A recent report (18) has indicated that B lymphocytes can secrete a cytokine(s) distinct from IL-2 that facilitates virus-specific cytolytic T-lymphocyte responses. It is possible that CLMF may have been the cytokine active in those studies. Thus, although B lymphocytes have not traditionally been viewed as cytokine-producing helper cells, it is conceivable that CLMF production constitutes a novel mechanism whereby B lymphocytes contribute to the amplification of T-lymphocyte responses. In addition to the biologic activities described in this report, CLMF by itself has been shown (i) to activate NK cells in an 18–22 hr assay, (ii) to facilitate the generation of specific allogeneic CTL responses, and (iii) to stimulate the secretion of γ interferon by resting peripheral blood lymphocytes (M.K.G., unpublished results). It can also synergize with low concentrations of recombinant IL-2 in the latter two assays and in causing the proliferation of resting peripheral blood lymphocytes. In view of its production by peripheral blood lymphocytes and its diverse actions on lymphoid cells, it appears that CLMF constitutes a new interleukin. We propose that CLMF be

given the provisional designation IL-12. The availability of recombinant CLMF will now make possible a broader and more detailed characterization of its biology.

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Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

Purpose: Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8$^+$ T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.

Patients and Methods: Patients were HLA-A2-positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.

Results: Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8$^+$ T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma-producing T cells against Melan-A (P = .015) after vaccination, but not against an Epstein-Barr virus control peptide (P = .86). There was a correlation between the magnitude of the increase in Melan-A-specific cells and clinical response (P = .046).

Conclusion: This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.

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M OST MELANOMA tumors express antigens that can be recognized by CD8$^+$ T cells.[1,2] Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.[3] Specific CD8$^+$ T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.[4]

The optimal immunization strategy for inducing tumor antigen-specific CD8$^+$ effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived[5,6] and bone marrow-derived[7] dendritic cells (DCs) have been loaded with

melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN-γ)–producing cytolytic effector cells.[8-10] Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors[11,12] and provision of exogenous IL-12, either alone[13] or combined with tumor antigen-based vaccines,[14-19] can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.[20] The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.[21] The effective range of doses indicated that a straight dose of 4 μg might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1[3] peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2-positive patients with

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advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

PATIENTS AND METHODS

Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rhIL-12.[4] The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2-positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status ≥70, and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, Candida, and Trichophytin, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis. 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.[22] Only 45% were positive for at least one recall antigen (mumps, Candida, or Trichophytin) by DTH skin testing.

RT-PCR Analysis

RNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.[21] Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide-stained agarose gel. No formal quantitation was performed.

Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rhIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 40×10^6 cells/mL. At least 10×10^6 cells from each sample were cryopreserved to prepare $CD8^+$ and $CD8^-$ fractions for subsequent correlative immunologic studies. The Melan-A$_{27-35}$ peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at

-80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20 μmol/L in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10 μmol/L; target number of PBMCs, 10^8) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from 78 to 100×10^6.

rhIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10 μg under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rhIL-12 was loaded into 3-mL syringes and used within 4 hours. rhIL-12 (4 μg) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rhIL-12 for each cycle.

Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

Table 1. Patient Characteristics

Patient Characteristic	No.	%
Age, years		
Median	58	
Range	35-79	
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2	None	
≥3	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy	None	
Adjuvant IFN-α	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN-α, interferon alfa-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

lesions; a partial response (PR) was defined as ≥ 50% reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

CD8+ T-Cell Preparation

CD8+ and CD8− fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. CD8+ T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound CD8− fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific CD8+ T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, CD8− cells were thawed from each time point and pooled, pulsed with 50 μmol/L Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with beta$_2$-microglobulin, irradiated (3,000 rad), washed, and plated at 2 × 10^6 cells/well in 24-well plates. CD8+ T cells were thawed and cultured with the irradiated CD8− cells at 4 × 10^5 cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A-pulsed irradiated CD8− cells. After an additional 5 days the cells were collected and tested.

ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15 μg/mL of antihuman IFN-γ antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and CD8+ T cells, either freshly thawed at 5 × 10^4 cells/well or after in vitro expansion at 5 × 10^3 cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing-deficient cell line, American Type Culture Collection no. CRL 1992) were pulsed for 1 hour at 37°C with 50 μmol/L peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of CD8+ T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5 μg/mL) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN-γ antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin-alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN-γ in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired t test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided t test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

RESULTS

Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

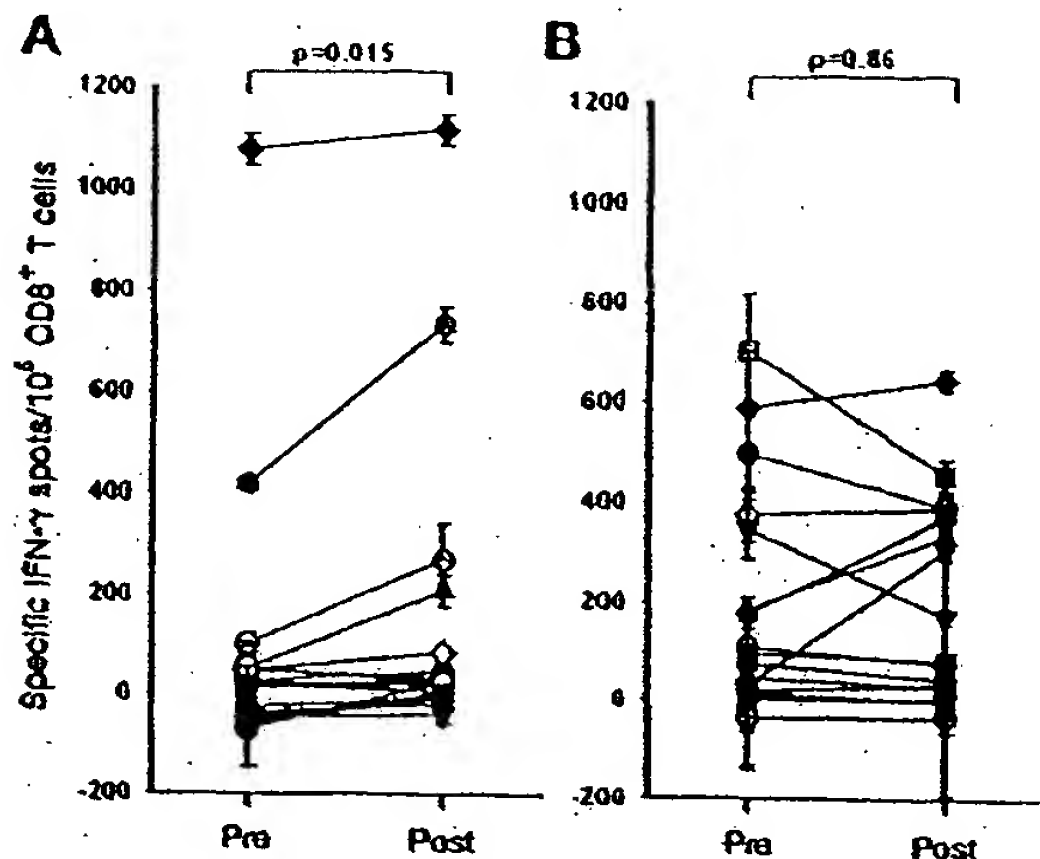| Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

Fig 1. Interferon gamma ELISpot frequencies by CD8+ T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired t test.
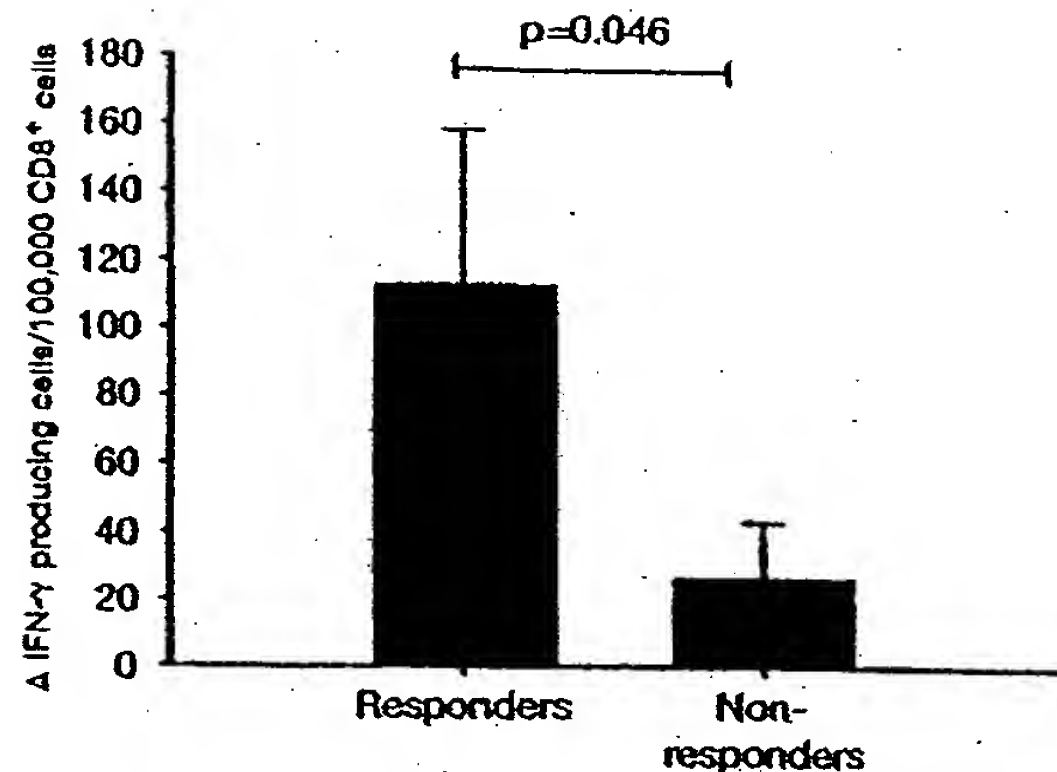

Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired t test.

Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN-γ ELISpot assay was used to monitor the immune response to immunization. Cryopreserved CD8+ T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A-specific CD8+ T cells before vaccination, with as high as 1% of CD8+ cells responding to this peptide. These T cells were functional because they produced IFN-γ. The majority of patients showed an increase in the frequency of Melan-A-specific cells after immunization (P = .015). In contrast, the frequencies of specific CD8+ T cells responding to the EBV peptide did not vary significantly overall (P = .86). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A-specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A-specific T cells for the clinical responders was 112 ± 45 and for nonresponders was 26 ± 16, indicating that a greater absolute increase in Melan-A-specific T cells was associated with tumor regression (P = .046).

Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known

negative prognostic factor,[23] survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH (P = .005). In addition, the median survival for patients who experienced a significant increase in Melan-A-specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A-specific cells (Fig 3C; P = .120).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A-specific CD8+ T cells before immunization correlated with either outcome. The median pretreatment Melan-A-specific T cell frequency was 23 in clinical nonresponders and −26 in responders. To increase the sensitivity of the assay to detect Melan-A-specific T cells, an in vitro expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A-specific T cell frequencies after in vitro expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A-specific T cells and clinical outcome.

Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A-negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for
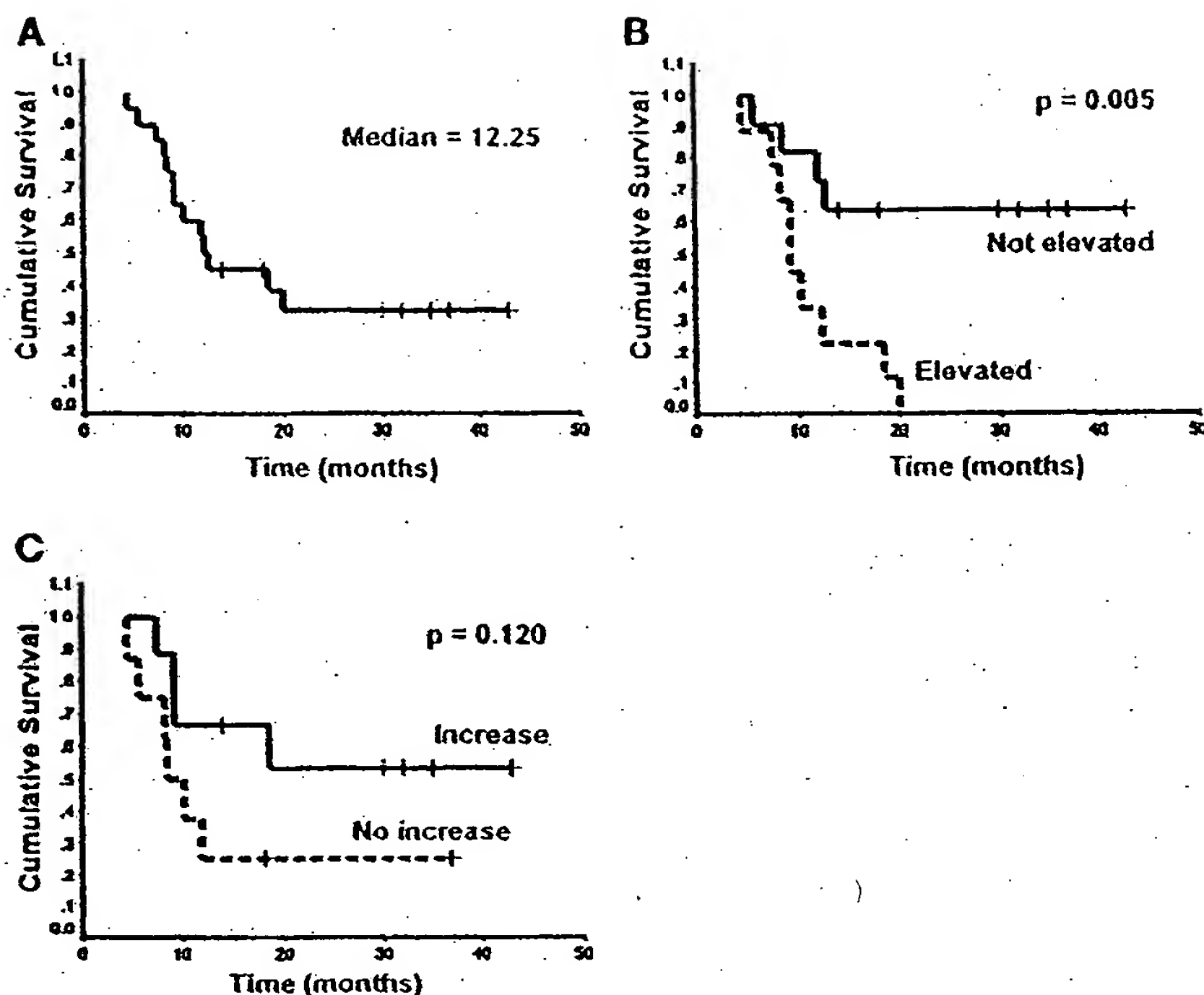
A

B

C

Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing CD8⁺ T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2-positive patients with advanced melanoma. We observed a significant increase in Melan-A-specific IFN-γ-producing CD8⁺ T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended in vitro culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results

support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.[24]

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved CD8⁺ T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A-reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified CD8⁺ T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of CD8⁻ T cells, CD4⁺ T cells, and natural killer T cells that could have produced IFN-γ in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A-specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (P)	Correlation With Survival (P)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pre†	.290	.644
Strong Melan-A pre-Rx†	.644	.481
Increased Melan-A post versus pre†	.046	.120
Strong in vitro expansion of Melan-A§	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two sided), except the differences between pre- and posttreatment, which were determined using an unpaired t test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

*At least 90 spots per 10^5 CD8$^+$ T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired t test between clinical responders and nonresponders.

‡At least 40 spots per 10^5 CD8$^+$ T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per 10^5 CD8$^+$ T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous CD8$^-$ cells and IL-2.

PBMC + rhIL-12 and in other trials[21,25] in which in vitro expansion had been performed before assessment of IFN-γ production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells in vivo.

High frequencies of Melan-A–specific, IFN-γ–producing CD8$^+$ T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A–specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,[26] those cells had a naïve surface phenotype and did not produce high levels of IFN-γ. What did correlate with clinical response in our current study is a meaningful increase in Melan-A–specific T cells posttreatment. These increases were modest (a net gain of 112 spots per 10^5 CD8$^+$ T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in
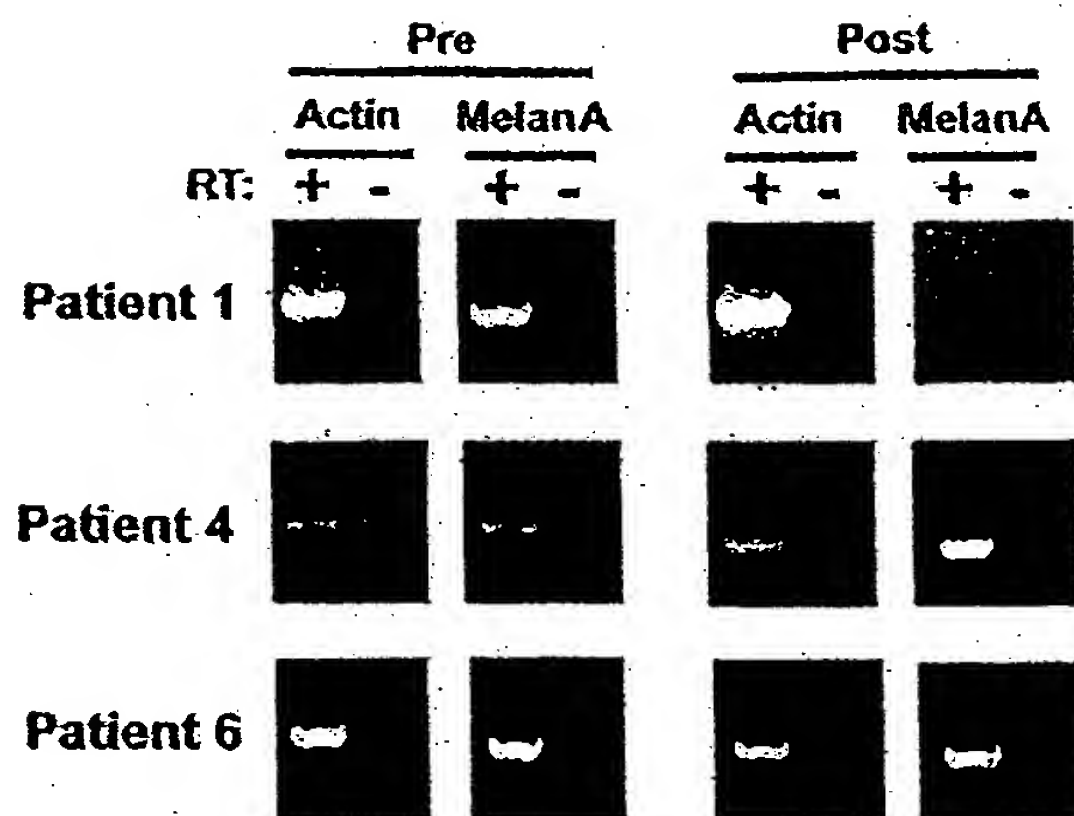
Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

T-cell frequencies using standard assays have been observed in other studies.[27]

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A–specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies[28] and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.[29,30] Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells in vivo.

ACKNOWLEDGMENT

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Vaccination with Mage-3A1 Peptide–pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

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Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin, 3×10^6 DCs each subcutaneously and intradermally, followed by two intravenous injections of 6×10^6 and 12×10^6 DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1–specific CD8+ cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and CD8+ T cell infiltration, whereas nonregressing lesions lacked CD8+ T cells as well as Mage-3 mRNA expression. This study proves the principle that DC "vaccines" can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active CD8+ CTL–tumor cell interaction in situ as well as escape by lack of tumor antigen expression.

Key words: dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

It is now established that the immune system has cells, particularly CD8+ CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),[1] "nature's adjuvant" for eliciting T cell immunity (3). Another is that

tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimelanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

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[1]Abbreviations used in this paper: CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.

antigens but lack full T cell–stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen–loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, immature DCs have been employed (9–11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of *mature* and stable DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide–pulsed mature DCs reliably enhance Mage-3A1–specific CD8$^+$ and recall CD4$^+$ T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1–specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC-mediated immunization and were accompanied by CD8$^+$ T cell infiltration, we propose that the induced Mage-3A1–specific CTLs are active in vivo.

Materials and Methods

Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/Union Internationale Contre Cancer pTNM staging system) that was not curable by resection and was progressive despite chemo(immuno)therapy. Further inclusion criteria were an expected survival ≥4 mo, Karnofsky index ≥60%, age ≥18 yr, measurable disease, HLA-A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosurea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was *not* required. Important exclusion criteria were active central nervous system (CNS) metastasis, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen/paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, NY) and performed under supervision of its Office of Clinical Trials Management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits 1, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF-α had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis (≥10^{10} nucleated cells) were isolated on Lymphoprep™ (Nycomed Pharma) and divided into three fractions. The first fraction of 10^9 PBMCs was cultured on bacteriological petri dishes (Cat. #1005; Falcon Labware) coated with human Ig (100 μg/ml; Sandoglobin™; Sandoz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 20 μg/ml gentamicin (Refobacin 10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of 3 × 10^8 PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test I. Adherent monocytes were cultured in 1,000 U/ml GM-CSF (10 × 10^7 U/mg; Leukomax™; Novartis) and 800 U/ml IL-4 (purity >98%; 4.1 × 10^7 U/mg in a bioassay using proliferation of human IL-4R$^+$ CTLL; CellGenix; expressed in *Escherichia coli* and produced under good laboratory practice conditions but verified for good-manufacturing practice [GMP] safety and purity criteria by us) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP-rhu TNF-α (purity >99%; 5 × 10^7 U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15) (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the

Multitest Merieux was >10 mm; both purchased from the Bacterial Vaccines Department of the Statens Serum Institute, Copenhagen, Denmark). The recall antigen was added at 10 µg/ml for the last 24 h, and the Mage-3A1 peptide was added at 10 µM directly to the cultures for the last 8 h (if immunity to recall antigens was strongly boosted, the dose of recall antigen was reduced to 1.0 or 0.1 µg/ml or was omitted for the intravenous DC injections to avoid a cytokine release syndrome). On day 7, mature DCs were harvested, resuspended in complete medium, washed, and pulsed once more with Mage-3A1 peptide (now at 30 µM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but no recall antigen); DCs that served as negative control in the DTH tests were not pulsed at all. An aliquot of the DCs to be used for vaccinations was analyzed as described (13) to assure that functionally active and mature DCs were generated. The features of the DCs are described in Results. Release criteria were typical morphology (>95% nonadherent veiled cells) and phenotype (>95% HLA-DR^{+++}CD86^{+++}CD40^{+}CD25^{+}CD14^{-} and >65% homogenously CD83^{++}).

Immunization Schedule

A total of five vaccinations (three into the skin followed by two intravenously) with antigen-pulsed DCs were given at 14-d intervals (Table II). This design was chosen to explore the toxicity and efficacy of various routes in this trial. For vaccinations 1–3, 3×10^6 DCs were given subcutaneously at two sites (1.5×10^6 DCs in 500 µl PBS per site) and 3×10^6 intradermally at 10 sites (3×10^5 DCs in 100 µl PBS per site). The injection sites were the ventromedial regions of the upper arms and the thighs close to the regional lymph nodes and were rotated clockwise. Limbs where draining lymph nodes had been removed and/or irradiated were excluded. For intravenous vaccinations 4 and 5, a total of 6 and 12×10^6 antigen-pulsed DCs (resuspended in 25 or 50 ml PBS plus 1% autologous plasma) was administered over 5 and 10 min, respectively. Premedication with an antipyretic (500 mg acetaminophen/paracetamol p.o.) and an antihistamine (2.68 mg clemastinhydrogenfumarat i.v.) was given 30 min before intravenous DC vaccination.

Evaluation of Immune Status

Recall Antigen–specific Proliferation and Cytokine Production. PBMCs were cultured in triplicate at two dose levels (3×10^4 and 1×10^5 PBMCs/well) plus or minus TT or tuberculin (at 0.1, 1, and 10 µg/ml) and pulsed on day 5 with [^{3}H]thymidine for 12 h. In all cases, the highest cpms were obtained with the highest doses of PBMCs and antigen and are shown in Fig. 2. IL-4 and IFN-γ levels were measured in culture media by ELISA (Endogen, Inc.). In a separate plate, staphylococcal enterotoxin (SEA; Serva) was added at 0.5, 1, and 5 ng/ml, and proliferation was assessed after 3 d to provide a positive control for helper T cell viability and responsiveness.

Enzyme-linked Immunospot Assay for IFN-γ Release from Single Antigen-specific T Cells. To quantitate antigen-specific, IFN-γ–releasing Mage-3A1-specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (16). PBMCs (10^5 and 5×10^5/well) or in some cases CD8$^+$ or CD4$^+$ T cells (isolated by MACS™ according to the manufacturer, Miltenyi Biotec) were added in triplicate to nitrocellulose-bottomed 96-well plates (MAHA S4510; Millipore Corp.) precoated with the primary anti–IFN-γ mAb (1-D1K; Mabtech) in 50 µl ELISPOT

medium (RPMI 1640 and 5% heat-inactivated human serum) per well. For the detection of Mage-3A1–reactive T cells, the APCs were irradiated T2.A1 cells (provided by P. van der Bruggen, Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed with MHC class I–restricted peptides (Mage-3A1 peptide and the HIV-1 p17-derived negative control peptide GSEELRSLY) added at 7.5×10^4/well (final volume 100 µl/well). After incubation for 20 h, wells were washed six times, incubated with biotinylated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, and stained with Vectastain Elite kit (Vector Labs.). For detection of TT-reactive T cells, TT was added at 10 µg/ml directly to the PBMCs (1 or 5×10^5 PBMCs/flat-bottomed 96-well plate). Assays were performed on fresh PBMCs. Spots were evaluated and counted using a special computer-assisted video imaging analysis system (Carl Zeiss Vision) as described (16).

Semiquantitative Assessment of CTL Precursors. The multiple microculture method developed by Romero et al. (17) was used to obtain a semiquantitative assessment of CTLp (precursors) specific for Mage-3A1 peptide. Aliquots of frozen PMBCs were thawed and assayed together. CD8$^+$ T cells were isolated with magnetic microbeads (MACS™ separation columns; Miltenyi Biotec) and seeded at 10^4/well in 96-well round-bottomed plates in RPMI 1640 with 10% heat-inactivated human serum. The CD8$^-$ PBMCs were pulsed with peptide Mage-3A1 or the influenza PB1 control peptide VSDGGPNLY (10 µg/ml; 30 min at room temperature), irradiated (30 Gy from a cesium source), and added as an APC population at 10^5/well together with IL-2 (10 IU/ml final) and IL-7 (10 ng/ml final) in a total volume of 200 µl/well. On day 7, 100 µl fresh medium was substituted, and peptide Mage-3A1 or PB1 (1 µg/ml final) and IL-2 (10 U/ml) was added. On day 12, each microwell was divided into three equal samples to test cytolytic activity in a standard 4-h ^{51}Cr-release assay on peptide-pulsed (10 µg/ml for 1 h at 37°C) T2A1 cells, nonpulsed T2A1 cells, and K562 target cells, respectively. All of the assays were performed with an 80-fold excess of nonlabeled K562 to block NK activity. Microwells were scored positive if lysis of T2A1 targets with peptide minus lysis without peptide was ≥12% and this specific lysis was greater than or equal to twice the lysis of T2A1 targets without peptide plus six as described (18). We aimed at testing 30 microwells of 10^4 CD8$^+$ T cells. Therefore, 1/30 positive wells equals at least one CTLp in 3×10^5 (i.e., 30 wells at 10^4 CTLp per well) CD8$^+$ T cells (corresponding to ~3×10^6 PBMCs).

DTH. DTH to Mage-3A1 peptide was assessed by intradermal injection at two sites of each 3×10^5 Mage-3A1 peptide–loaded DC in 0.1 ml PBS. Negative controls were nonpulsed autologous DCs in 0.1 ml PBS and 0.1 ml PBS. DTH to seven common recall antigens (Multitest Merieux) including TT and tuberculin was performed on visits 1, 5, and 8 (Table II).

Assessment and Analysis of Tumor Tissue

For recruitment into the study, Mage-3 gene expression in at least one metastatic deposit had to be demonstrated by RT-PCR as described (14). Accessible superficial skin metastases were removed whenever possible after the vaccinations and subjected to Mage-3 RT-PCR as well as routine histology and immunohistology (to characterize cellular infiltrates).

Statistical Analysis

For analysis of the immune response, pre- and postimmunization values were compared by paired *t* test after logarithmic transformation of the data. Significance was set at $P < 0.05$.

Results

Patient Characteristics

All 13 patients were HLA-A1+, had proven Mage-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemoimmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in

appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR+++CD86+++CD40+CD25+CD14−), and acted as strong stimulators of an MLR at DC/T cell ratios of ≤1:300 (13). Most (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with Mage-3A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. Patients' Characteristics, Status before DC Vaccination, and Response to DC Vaccination

Patient code	Sex-Age	Onset stage IV	Previous therapy	Metastases at study entry* regional skin	LN	Skin	distant LN	Lung	Liver	Other	Clinical Response 14 days after the 5th vaccination	Survival
Patients with objective tumor regression											
04	M48	1/98	PCI				▨	▨		▨	complete regression of all but 1 lung metastasis, overall progression	10 + >9
06	F61	10/97	CI			▨		▨	▨		complete regression[e] of 1 lung + 4 s.c.[Δ] metastases, overall progression	6 + >16
07	F48	6/97	C			▨				▨	complete regression[e] of 1 lung[a] + 2 s.c.[Δ] metastases, overall progression	13 + 12†
08	M67	11/97	PC		▨		▨	▨			complete regression[c] of lung+liver+4 s.c.[Δ] metastases, overall progression[b]	8 + 3†
09	F43	5/98	C				▨		▨		Partial regression of 1 lung metastasis, overall progression	4 + >11
12	M54	9/96	CI			▨	▨				partial regression of axillary LN metastases, overall progression	26 + >9
Patients without objective tumor regression											
02	F73	5/96	PCI	▨		▨	▨	▨		▨	continuous progression	18 + 5†
05	F49	10/97	CI		▨		▨	▨			continuous progression	5 + >17
10	M62	8/98	C	▨		▨					continuous progression	1 + 6†
11	F72	7/98	C		▨	▨		▨		▨	continuous progression	4 + 9†
13	M34	12/97	CI				▨	▨		▨	continous progression	12 + 5†

Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen.
Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed.
Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases).
Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. †Patient deceased.
*CNS metastases were regressing at study entry after gamma knife treatment.
Δ Developed (in part) after study entry.
°Determined by autopsy.
▴Sudden death from bleeding into CNS metastasis on visit 8.
eThe regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8.
mediast., mediastinum; pancr., pancreas.

Table II. *Study Design*

Activities	Screen	Leuka pheresis	Vacc. #1 3 Mio s.c. 3 Mio i.d.	Vacc. #2 3 Mio s.c. 3 Mio i.d.	Vacc. #3 3 Mio s.c. 3 Mio i.d.	Vacc. #4 6 Mio i.v.	Vacc. #5 12 Mio i.v.	Final Evaluation
Clinical visit	1	2	3	4	5	6	7	8
Day	-28/-14	-9	+1	+14	+28	+42	+56	+70
Vaccination			X	X	X	X	X	
Multitest Merieux	X				X			X
DTH to Mage-3A1 peptide-loaded DC			X		X		X	
Recall antigen proliferation		X						X
CTLp analysis		X				X		X
ELISPOT Mage-3A1		X	X	X	X	X	X	X
ELISPOT recall antigen		x	x	x	x	x	x	x

X, prespecified in the protocol as obligatory; x, optional.

neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration >10 mm in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to 40°C) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63% and by sonography in 83% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

Immunological Responses

Boosting of Recall Antigen-specific Immunity. PBMCs that had been frozen before vaccination and 14 d after vaccination 5 were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) ($P < 0.004$; Fig. 2). Supernatants from the proliferative assays contained large amounts of IFN-γ (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN-γ/IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN-γ ELISPOT analysis. We found an increase after the intracutaneous vaccinations ($P < 0.02$) but a peculiar decrease after the intravenous vaccinations ($P < 0.008$; Fig. 3). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.
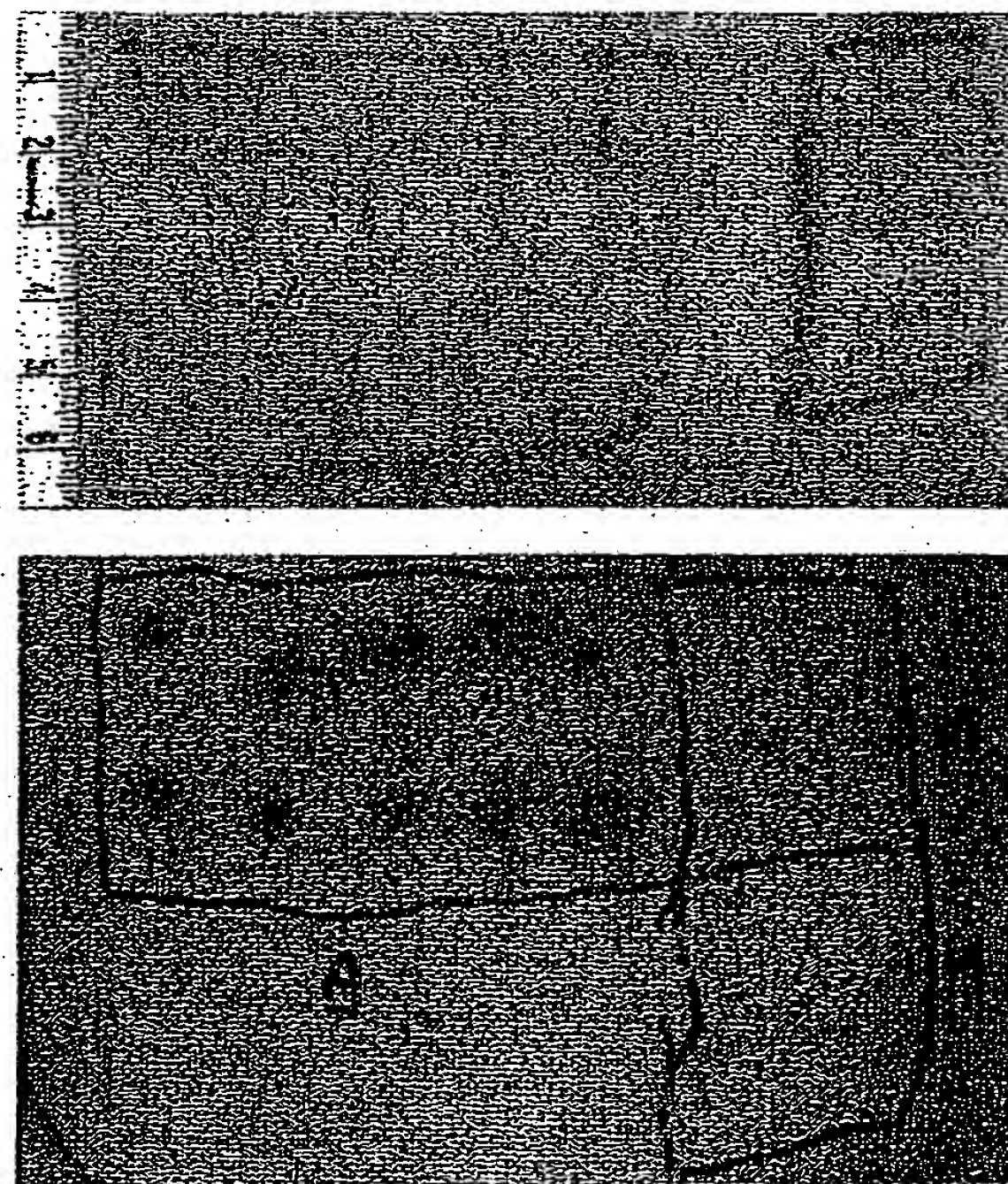
Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the 10 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration >10 mm in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).
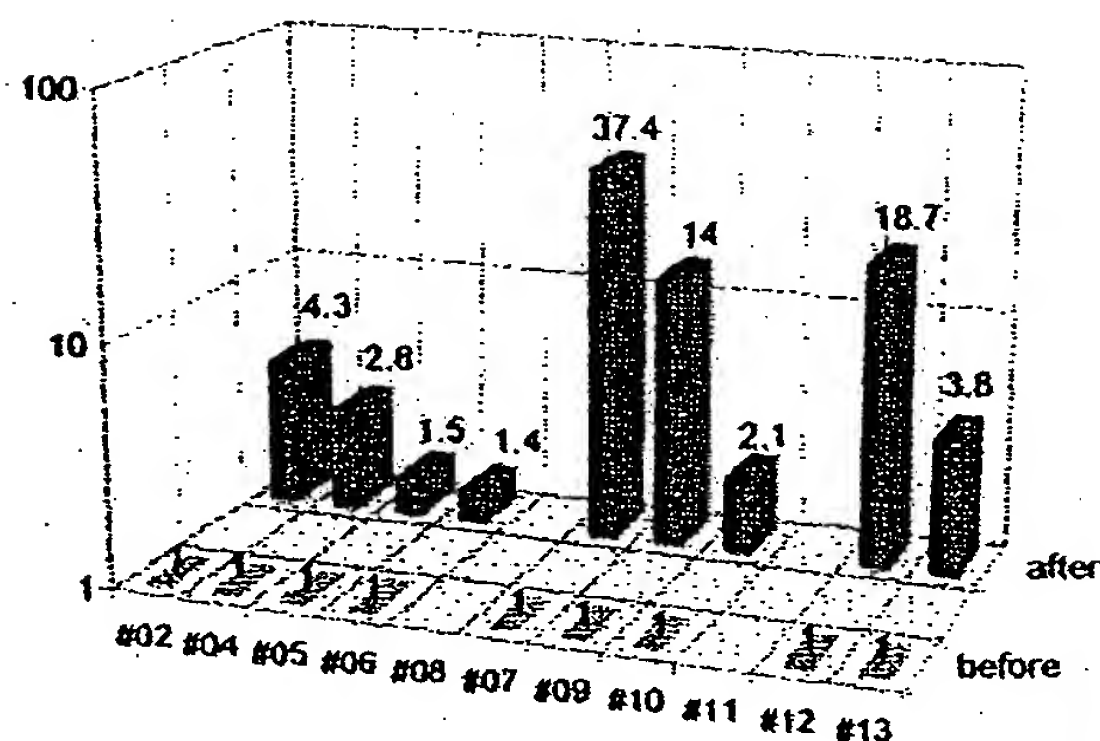
Figure 2. Recall antigen-specific immunity (tuberculin in patient 10; TT in all others) as assayed by antigen-specific proliferation. The cpm values determined after therapy (14 d after vaccination 5) are shown as multiples of pretherapy cpm values. Absolute cpm (cpm with recall antigen minus cpm without antigen) after therapy was 68,917 in patient 02, 85,225 in patient 04, 16,759 in patient 05, 7,913 in patient 06, 16,367 in patient 07, 107,923 in patient 09, 22,790 in patient 10, 4,507 in patient 12, and 1,831 in patient 13 (SEM for all measurements was <20%). Patients 08 and 11 could not be evaluated due to shortage of cells after therapy.

Expansion of Mage-3A1-specific CTLp. Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1-specific CTLp as indicated by the increase (mean, eightfold; $P < 0.008$) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ($P < 0.0013$) but then decreased after the two additional intravenous vaccinations in all but one of these patients ($P < 0.026$). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 influenza peptide that served as a specificity control (not shown).

ELISPOT Analysis for IFN-γ-releasing Mage-3A1-specific T Cells. We also tried to detect Mage-3A1-specific CTL effectors in uncultured fresh, nonfrozen PBMCs by performing ELISPOT analyses at 14-d intervals on all patients. A significant increase of Mage-3A1-reactive IFN-γ spot-forming cells was apparent only in patients 07 and 09 after the first and second vaccinations, respectively, but the frequency of Mage-3A1-specific effectors was very high (~5,000 and 10,500/10⁷ CD8⁺ T cells; not shown).

DTH Test to Mage-3A1 Peptide-loaded DCs. Tests of DTH to Mage-3A1 peptide-loaded DCs yielded erythema and/or induration (>5 mm diameter) in 7/11 patients (not shown). The results were, however, equivocal due to the frequently observed background to nonpulsed DCs (up to 10 mm in diameter) and the variability from test site to test site.

Clinical Responses

At the end of the trial, i.e., ~2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07), it was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of CD8⁺ T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 mRNA⁺. However, all of the samples removed at the end (two and six, respectively) were Mage-3 mRNA⁻, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of CD8⁺ T cells was not found in any of these lesions.

Discussion

In deciding on the source of DCs for this phase I trial, we selected *mature*, monocyte-derived DCs for the follow-

Figure 3. Kinetic analysis of immunity to recall antigens as assessed by TT-specific IFN-γ ELISPOT (SEM for all measurements was <20%). Blood was drawn (see Table II, Study Design) before the first DC vaccination and then every 14 d just before administration of the next DC vaccination (e.g., pre Vacc # 2 means immediately before vaccination 2, i.e., 14 d after vaccination 1), and finally after therapy. Time points at which vaccinations were not performed lack bars. Note the increase after the intracutaneous vaccinations and the decline upon the two vaccinations after intravenous ones. Patient 10, who received tuberculin-pulsed DCs, exhibited no significant change in the TT-specific IFN-γ ELISPOT as expected.

Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axis and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

ing reasons. Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses in vitro (reference 22 and Jonuleit, H., A. Gieseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in

lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1-specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per 10^7 CD8$^+$ T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 CD8$^+$ T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1-specific T cells are CD8$^+$
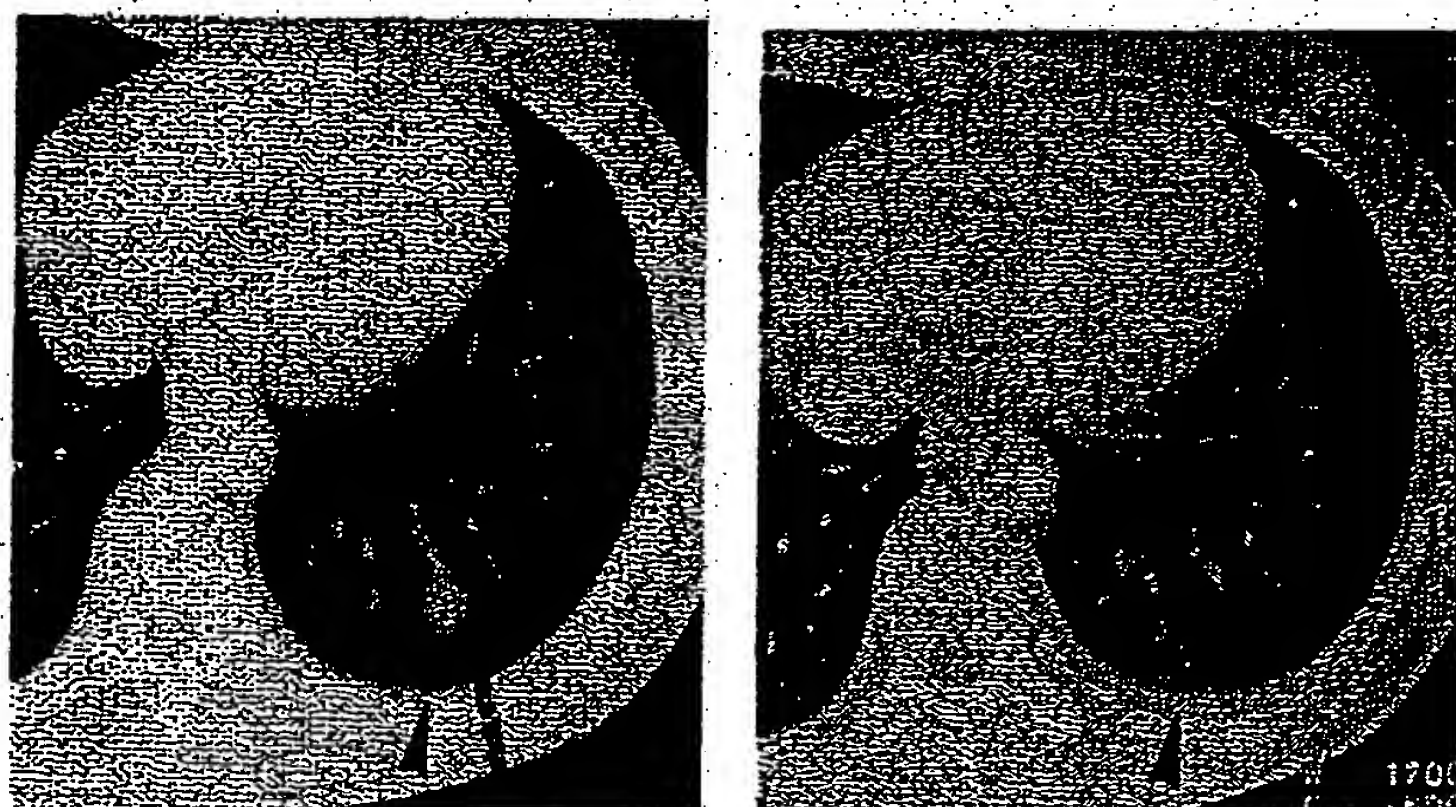
Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.
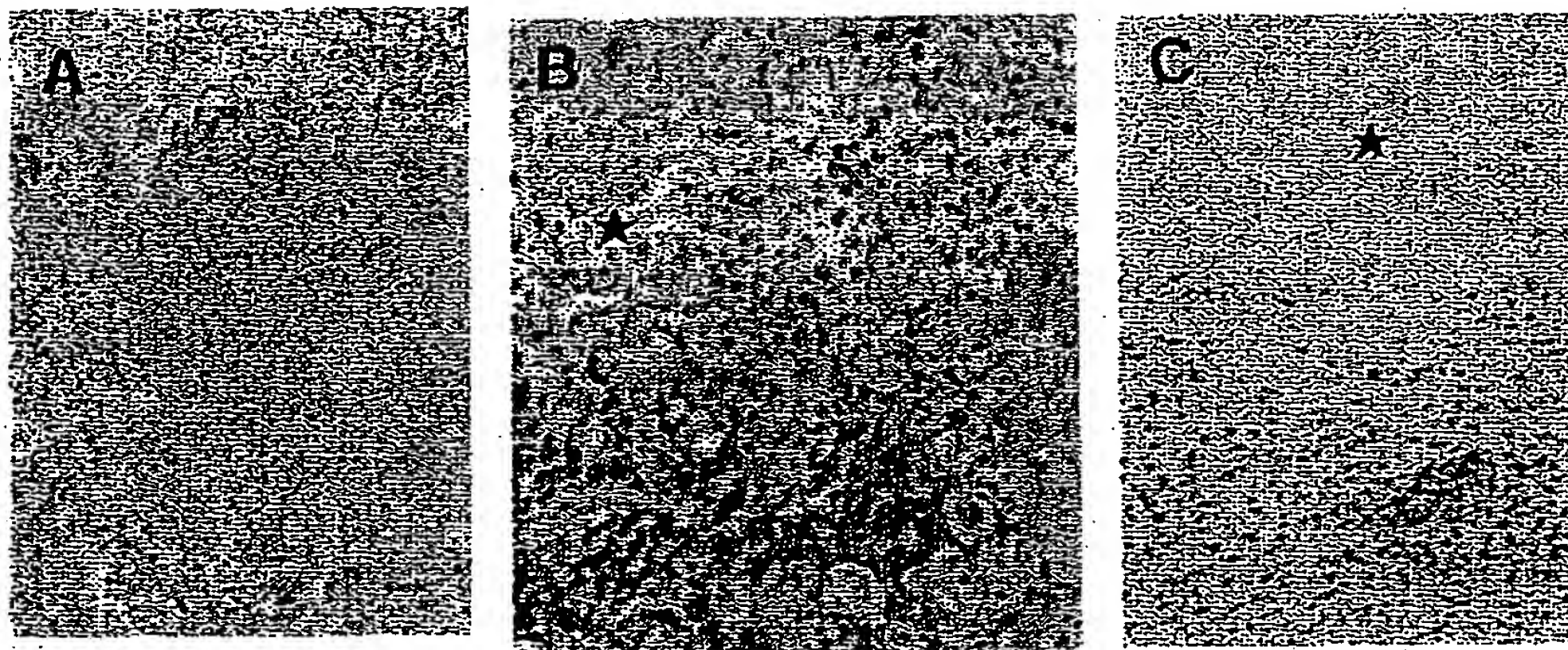
Figure 6. Regressing subcutaneous metastases in patient 06 display a CD8$^+$ lymphocytic infiltrate (alkaline phosphatase/antialkaline phosphatase immunohistochemical staining with anti-CD8 mAb) that surrounds (A) and infiltrates (B) the tumor. Areas of damaged (B, ★) and necrotic (C, ★) melanoma cells are obvious in the vicinity of the CD8$^+$ T cell infiltrate. The metastasis expressed Mage-3, as demonstrated by RT-PCR (data not shown). Magnifications: A, 100; B, 250; C, 160.

T cells and DTH assays typically detect primed CD4$^+$ T cells, we suspect that DTH to MHC class I peptide–pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific CD8$^+$ T cell–mediated immunity.

In contrast, we found sizable expansions of Mage-3A1-specific CTL precursors in PBMCs from a majority (8/11) of patients ($P < 0.008$; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1-specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies (>5,000/10^7 CD8$^+$ T cells). Perhaps active CD8$^+$ effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations ($P < 0.0013$) and then decreased ($P < 0.026$) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3-reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were all progressive despite standard chemotherapy and even chemoimmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1-specific CTLs. This interpretation is supported by the heavy infiltration with CD8$^+$ T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 mRNA$^-$ (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen–negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1-specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(immuno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between

CD8+ CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing variables such as DC maturational state, route, dose, and schedule or by improving the short life span of DCs in vivo (31, 32); at the level of the T cell, e.g., by providing melanoma-specific CD4+ T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

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Immunomodulation by an Inhibitor of S-Adenosyl-L-Homocysteine Hydrolase: Inhibition of *in Vitro* and *in Vivo* Allogeneic Responses

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The response of murine T cells to MHC class II determinants on allogeneic cells induces helper T cell activation and the development of cytotoxic T cells. We have recently established that an S-adenosyl-L-homocysteine hydrolase inhibitor, (Z)-5'-fluoro-4',5'-didehydro-5'-deoxyadenosine (MDL 28,842), is a potent immunosuppressive agent which selectively inhibits T cell activation. In this report we characterize the effect of MDL 28,842 on *in vitro* and *in vivo* models of transplant rejection. *In vitro*, MDL 28,842 inhibited the generation of cytotoxic T cells in the murine mixed lymphocyte reaction with an IC_{50} of less than 0.1 μM. MDL 28,842 (1.0 μM) totally inhibited the generation of cytotoxic T cells when added up to 3 days after the initiation of culture with no apparent cell toxicity. *In vivo*, MDL 28,842 given by gavage at 5.0, 2.5, or 1.0 mg/kg/day inhibited the increase in popliteal lymph node weight induced by injection of allogeneic spleen cells into the footpad. MDL 28,842 was also evaluated in a model of graft rejection. Skin allografts on animals given MDL 28,842 at 5 mg/kg/day (ip) for the first 6 days following transplantation survived for 12.2 days, compared to 8.7 days for control animals. Cyclosporin A (CSA) given at 5.0 mg/kg/day did not prolong graft survival. The combination of MDL 28,842 and CSA was not any more effective than MDL 28,842 alone. Based on these findings, we suggest that MDL 28,842 is useful in the prevention of allograft rejection. © 1993 Academic Press, Inc.

INTRODUCTION

The immune response of T cells to foreign MHC determinants is the basis for transplant rejection (1). Agents which inhibit T cell activation, such as cyclosporin A (CSA) and FK-506, are effective in preventing allogeneic graft (allograft) rejection (2, 3). We have recently demonstrated that a potent irreversible mechanism-based inhibitor of S-adenosyl-L-homocysteine hydrolase (AdoHcyase), (Z)-5'-fluoro-4',5'-didehydro-5'-deoxyadenosine (MDL 28,842) (4, 5), selectively inhibits T cell activation and the production of interleukin-2 (6). Although the precise mechanism by which this compound affects T cells is unknown, MDL 28,842 increases the intracellular concentration of S-adenosyl-L-homocysteine (AdoHcy), produced during the metabolism of S-adenosylhomocysteine (AdoMet) (7). AdoHcy is a feedback inhibitor of the AdoMet-dependent transmethylation of a variety of biomolecules, among them nucleic acids

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and proteins (8–10), and therefore could affect lymphocyte activation. In humans, a congenital deficiency in another enzyme in the pathway of AdoMet metabolism, adenosine deaminase (ADA), results in severe immunodeficiency, with the most consistent feature being T cell dysfunction (11–13). MDL 28,842 inhibits T cell activation without the toxicity associated with inhibition of ADA (14, 15).

In vivo, we have recently demonstrated that MDL 28,842 is effective in two T cell-mediated experimental animal models of arthritis, collagen-induced arthritis in mice, and adjuvant arthritis in rats (16). In the present study, we demonstrate that MDL 28,842 inhibits the development of cytotoxic T cells in the mixed lymphocyte reaction, an *in vitro* correlate of transplant rejection. In addition, we demonstrate that MDL 28,842 inhibits the T cell response in the draining lymph nodes of animals immunized with allogeneic cells. In addition, MDL 28,842 is more effective than low-dose CSA in prolonging allogeneic skin graft survival in mice.

MATERIALS AND METHODS

Animals. Inbred mice, C57BL/6 (H-2^b), C3H/HeJ (H-2^k), DBA/2 (H-2^d), and BALB/C (H-2^d) were obtained from Jackson Labs (Bar Harbor, ME).

Reagents. CSA was obtained from Sandoz (Hanover, NJ). MDL 28,842 was synthesized at the Marion Merrell Dow Research Institute (4, 5).

Generation of cytotoxic T cells. Spleens were obtained from C57BL/6 and DBA/2 mice and single-cell suspensions made in Hanks' balanced salt solution (HBSS, calcium and magnesium free). Erythrocytes were lysed by treatment with Tris-buffered ammonium chloride (0.155 M NH$_4$Cl, 0.0165 M Tris, pH 7.2) at 37°C. The stimulator population (DBA/2) was irradiated (3000R) and both responders (3 × 10^7) and stimulators (3 × 10^7) were cocultured in T25 flasks at 37°C, 5% CO$_2$ in RPMI 1640 containing 10% FCS and 5 × 10^{-5} 2-ME (CM). MDL 28,842 was added at the beginning of culture or at times indicated. After 5 days, viable cells were recovered and assayed for cytotoxic effector cells in the ^{51}Cr release assay.

^{51}Cr release assay. P815 tumor cells (H-2^d) were used as targets. The cells were labeled with ^{51}Cr (10 μCi/10^6 cells) at 37°C, 5% CO$_2$ for 1 hr in CM. Washed target cells (10^4) were added to effector cells (in triplicate) in a 96-well round-bottom plate in a final volume of 100 μl. Effector:target cell ratios began at 25:1. The plate was centrifuged at 50g for 5 min and incubated at 37°C, 5% CO$_2$ for 4 hr. Target cells were incubated with CM alone for spontaneous ^{51}Cr release and with 1% SDS for maximal release. Following incubation, the plate was centrifuged once more, the supernatant fractions were collected, and released ^{51}Cr radioactivity was determined in a Beckman gamma counter. The percentage specific lysis is expressed as:

$$100 \times \frac{\text{Experimental cpm released} - \text{spontaneous cpm released}}{\text{maximal cpm released} - \text{spontaneous cpm released}}.$$

Fluorescence microfluorometry. CD8-positive cells were stained with a fluoresceinated anti-Lyt-2 antibody (Becton–Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C (Hialeah, FL) flow cytometer.

Popliteal lymph node assay. Spleen cells from BALB/C (H-2^d) and C57BL/6 mice obtained as described above were irradiated (2000R) and 10^7 cells from each strain

(in 50 μl PBS) were injected subcutaneously into each hind footpad. Seven days later animals were sacrificed and popliteal lymph nodes (LN) were removed and weighed. The net LN weight represents the weight of the LN draining the footpad injected with allogeneic cells minus the weight of the LN draining the syngeneic cell-injected footpad. MDL 28,842 at the indicated doses was given orally beginning 1 day prior to the spleen cell injections and continuing daily throughout the study.

Allogeneic skin transplantation. Skin from C57BL/6 or C3H/HeJ mice was transplanted onto BALB/C mice using a free skin grafting technique (17). Recipient animals were given compounds, intraperitoneally, 1 day before transplant, the day of transplant, and daily for either 6 days or until rejection of the graft. Mice were monitored daily for rejection. Rejection was determined by observation *in situ* and grafts were considered rejected when at least 50% of the graft's epithelium was visually destroyed. In addition, a representative graft from each group was prepared for histology and examined microscopically for rejection.

RESULTS

Effect of MDL 28,842 on the generation of cytotoxic cells in the mixed lymphocyte reaction. MDL 28,842 was added to mixed lymphocyte reaction cultures at the indicated concentrations at the initiation of culture. Five days later, viable cells were harvested and cytotoxic T cell generation was measured by the lysis of [51]Cr-labeled target cells. The results are presented in Fig. 1. MDL 28,842 was a potent inhibitor of cytotoxic T cell generation. The IC_{50} for this activity was less than 0.1 μM. The decreased cytotoxic T cell activity observed in cultures incubated with MDL 28,842 correlated with a decrease in the percentage of cells in the cultures expressing CD8,
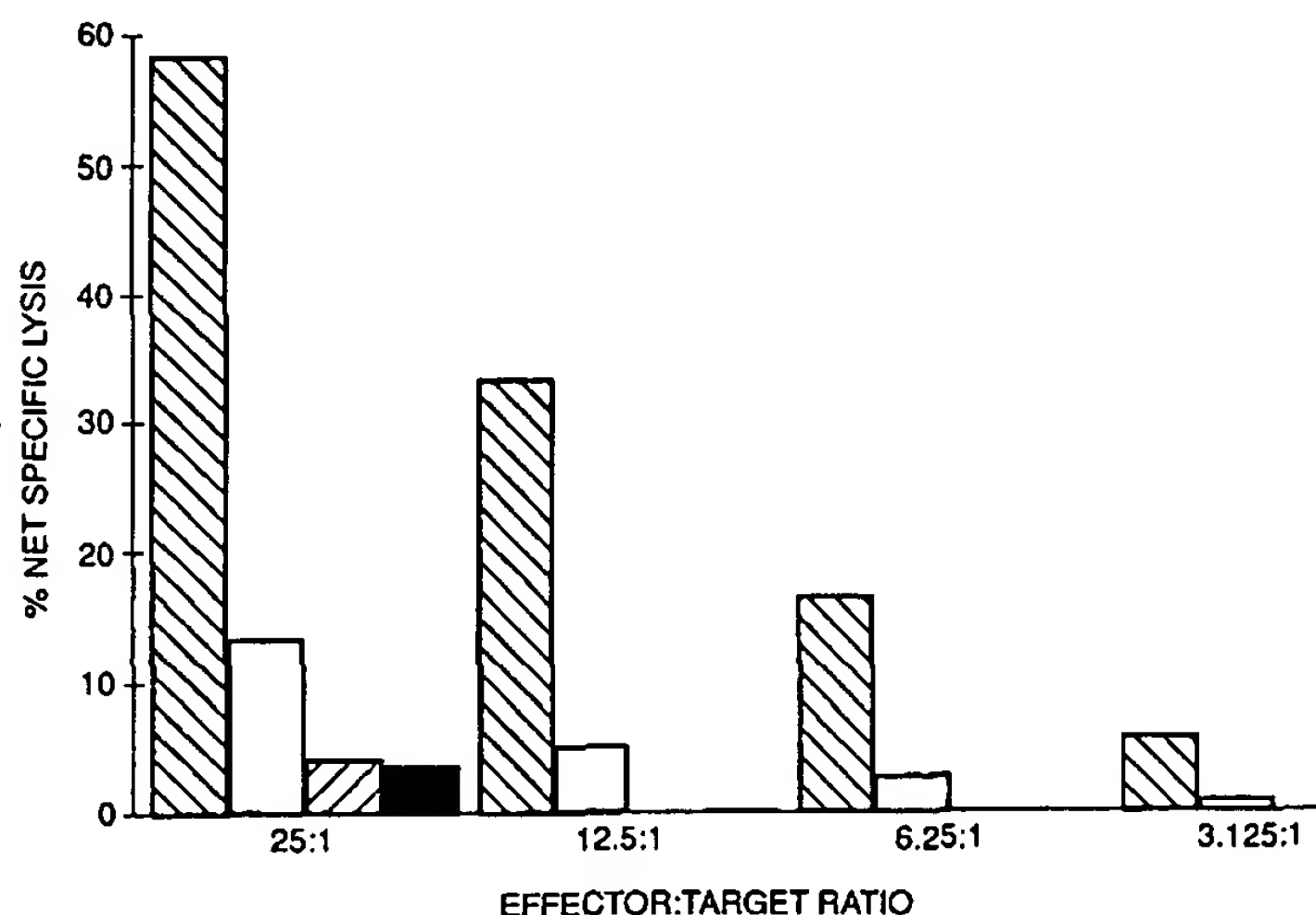
FIG. 1. Inhibition of cytotoxic T cell generation in the MLR by MDL 28,842. Cultures of C57BL/6 spleen cells (H-2[b]) and irradiated DBA/2 spleen cells (H-2[d]) were incubated in the absence of compound (▨), or in the presence of 0.1 (☐), 1.0 (▧), or 10 μM (■) MDL 28,842. Cytotoxic T cell activity against [51]Cr-labeled P815 tumor cells (H-2[d]) was measured 5 days later.

the cytotoxic T cell phenotype (data not shown). In subsequent experiments, MDL 28,842 was added after the initiation of culture to determine how late the compound could be added and still be effective. As shown in Table 1, the addition of MDL 28,842 (1.0 μM) up to 3 days after initiation of culture completely inhibited the generation of cytotoxic T cells. When the compound was added on Day 4 of the 5-day culture, cytotoxic T cell activity was observed, but it was substantially lower than the controls.

Inhibition of allogeneic response in the popliteal lymph nodes. BALB/C mice were dosed with MDL 28,842 by gavage at 5.0, 2.5, or 1.0 mg/kg. One day later they were injected in the footpad with 10^7 irradiated allogeneic (C57BL/6) spleen cells. The contralateral footpad received 10^7 irradiated syngeneic spleen cells. Mice were dosed with MDL 28,842 daily. Seven days after immunization, popliteal lymph nodes were isolated and weighed. In this assay, the weight correlates with the number of mononuclear cells and is related to the ongoing host versus graft T cell response (18, 19). The results are shown in Fig. 2. Treatment with all of the doses of MDL 28,842 inhibited the *in vivo* enlargement of the popliteal lymph nodes in response to allogeneic cells. Although the mean net weights observed in each treatment group suggests a dose-response, the groups were not significantly different. At the concentrations tested, all were potent inhibitors of this T-dependent response.

Effect of MDL 28,842 on skin allograft survival. Mice (8 per group) were given MDL 28,842 or CSA at 5.0 mg/kg (ip) beginning 1 day prior to skin transplantation and continuing for 6 days. The results are shown in Fig. 3. Control animals rejected skin grafts by an average of 8.7 days after transplantation. At 5 mg/kg, cyclosporin A had little effect. In contrast, MDL 28,842 prolonged skin allograft survival, with rejection delayed until 12.2 days after transplantation. Interestingly, mice given both MDL 28,842 and CSA rejected skin transplants earlier, on average, than mice given MDL 28,842 alone.

TABLE 1

Inhibition of the Development of Cytotoxic Effector Cells by MDL 28,842: Effect of Adding the Compound after Initiation of Culture

Experimental group[a]	Compound addition[b]	% Specific lysis[c]			
		Effector:target ratio			
		50:1	25:1	12.5:1	6.25:1
C57BL/6	—	0	0	0	0
C57BL/6 × DBA/2	—	52.9	65.3	52.9	25.2
C57BL/6 × DBA/2	Day 0	0	0	0	0
C57BL/6 × DBA/2	Days 1, 2, and 3	0	0	0	0
C57BL/6 × DBA/2	Day 4	30.4	25.7	10.1	1.3

[a] C57BL/6 spleen cells were incubated with irradiated DBA/2 spleen cells for 5 days at which time viable cells were harvested. Recovered cells were incubated for 4 hr at 37°C with ^{51}Cr-labeled P815 tumor cells (H-2^d).

[b] MDL 28,842 (1.0 μM) was added at the indicated times after the initiation of culture.

[c] C57BL/6 spleen cells were cultured with ^{51}Cr-labeled P815 tumor cells at the indicated effector:target ratios.
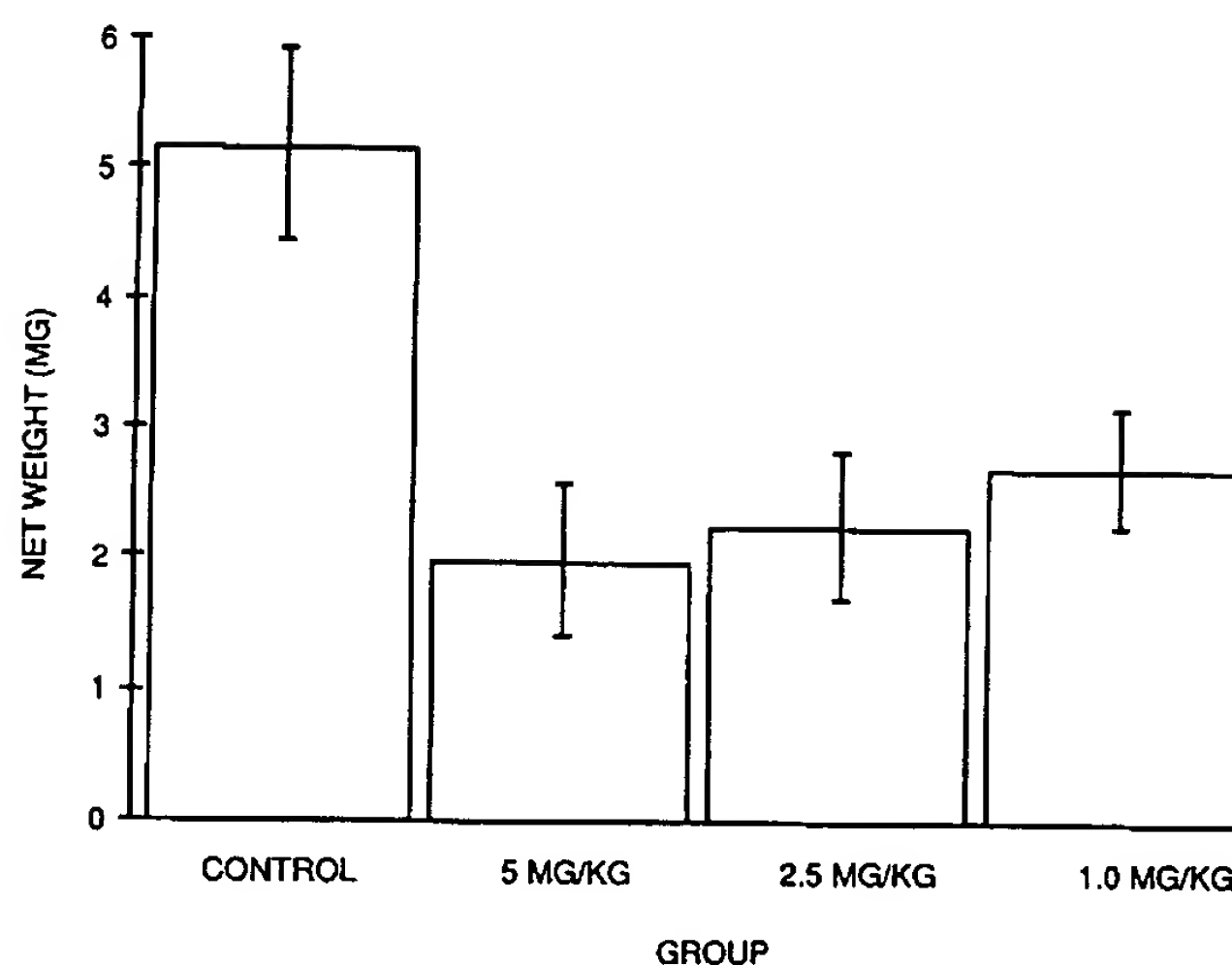
FIG. 2. MDL 28,842 inhibits lymph node enlargement stimulated by the transfer of allogeneic spleen cells. BALB/C mice (H-2^d) were injected with irradiated C57BL/6 spleen cells. Compound treatment was begun 1 day prior to injection (N = 6/group).

DISCUSSION

Inhibition of T cell activation, by agents such as cyclosporin A and, more recently, FK-506, has greatly increased the survival of tissue transplants (2, 3), although these compounds are not without side effects (20–22). MDL 28,842 is a compound which has been demonstrated to selectively inhibit mitogen-stimulated T cell activation *in vitro* and T-dependent antibody synthesis *in vivo* (6). In addition, MDL 28,842 is effective, both prophylactically and therapeutically, in the treatment of collagen-induced arthritis, a T cell-dependent experimental model of autoimmune disease (16). The precise mechanism by which MDL 28,842 mediates its effects is unclear. The compound was designed as a potent irreversible inhibitor of AdoHcyase, an enzyme in the pathway of *S*-adenosylmethionine metabolism (4, 5). Inhibition of AdoHcyase results in an accumulation of AdoHcy and the feedback inhibition of methyltransferases. These are involved in the methylation of a number of intracellular constituents, among them messenger RNA, important for cell activation (7–10). Lymphocytes seem to have a greater requirement than other cell types for methylation (23). There are other possible mechanisms by which inhibition of AdoHcyase could inhibit T cell activation. Depletion of homocysteine, the product of AdoHcyase-mediated reactions, would indirectly inhibit nucleotide synthesis by preventing the conversion of 5-methyltetrahydrofolate to tetrahydrofolate (24), which is required for folate-dependent purine and thymidylate synthesis (25). AdoHcy, which builds up in cells incubated with MDL 28,842, is also an inhibitor of PI synthase and therefore could inhibit second messenger signaling early in T cell activation (26). This possibility is not likely, since, in this report, we have shown that MDL 28,842 is effective in inhibiting the development of cytotoxic T cells *in vitro* when added as late as 3 days after the initiation of a 5-day mixed lymphocyte culture.
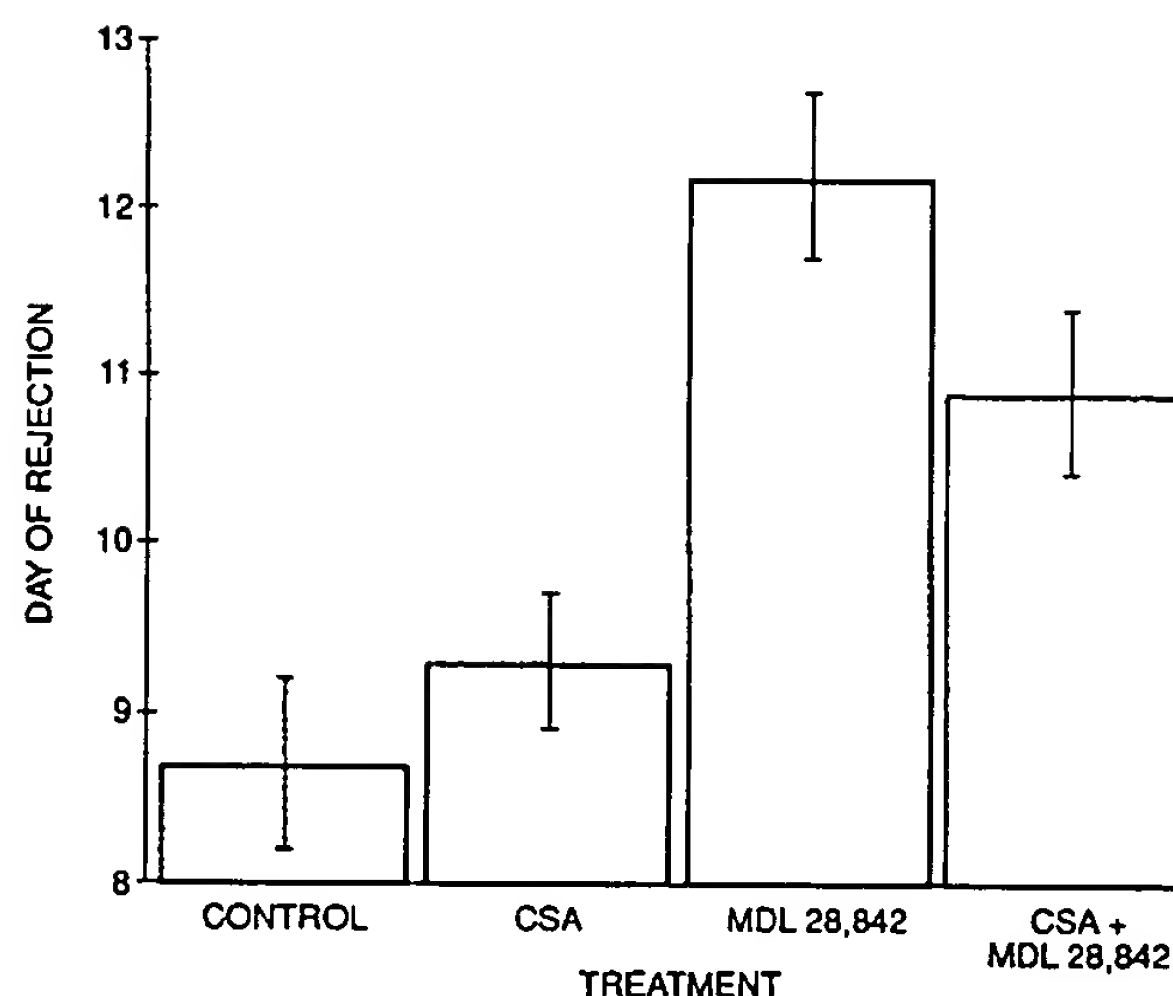
Fig. 3. MDL 28,842 prolongs allogeneic skin graft survival in mice. Comparison with CSA. The rejection of skin from C57BL/6 mice transplanted onto BALB/C mice was delayed in animals treated with MDL 28,842 at 5 mg/kg/day ip for 6 days compared to controls. CSA at 5 mg/kg/day ip did not prolong graft survival compared to controls and did not enhance the effect of MDL 28,842. (N = 8/group).

In vivo, MDL 28,842 also inhibited alloantigen-mediated T cell activation. The T cell dependent host versus graft response in the draining lymph nodes of mice injected in the footpads with irradiated allogeneic cells was reduced in animals treated with the compound. In addition, MDL 28,842 was more effective than CSA in prolonging allogeneic skin graft survival in mice. Preliminary studies suggest that MDL 28,842 also inhibits heart allograft rejection in rats (unpublished observations). The data presented previously (6, 16) and in this report demonstrate that MDL 28,842 is a potent immunosuppressive agent which inhibits T cell-mediated responses and may be useful in the prevention of transplant rejection.

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TEPOXALIN, A NOVEL IMMUNOMODULATORY COMPOUND, SYNERGIZES WITH CSA IN SUPPRESSION OF GRAFT-VERSUS-HOST REACTION AND ALLOGENEIC SKIN GRAFT REJECTION

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Tepoxalin, a dual 5-lipoxygenase and cyclooxygenase inhibitor with nonsteroidal antiinflammatory effects, has recently been shown to suppress NFκB transactivation and inhibit T cell proliferation via a mechanism very different from cyclosporine (CsA). In this report, we demonstrate that this novel immunosuppressive effect of tepoxalin is manifested in in vivo transplantation models. Tepoxalin suppressed murine spleen cell proliferation in a mixed lymphocyte reaction (MLR) with an IC_{50} of 1.3 μM. Coadministration of tepoxalin and CsA in MLR cultures showed an additive inhibitory effect. Oral administration of tepoxalin at 12 mg/kg/day to mice suppressed local graft-versus-host (GVH) responses by about 40% (n=10). Combination of tepoxalin and CsA at suboptimal doses synergized their immunosuppressive effects on GVH responses (n=20). In skin transplantation, the median survival time of allogeneic BALB/cByJ (H-2^d) mouse skin grafted onto C3H/HeJ (H-2^k) mice was 10.5 days (n=8), and was prolonged to 15.0 days (n=9) for recipient mice administered tepoxalin at 50 mg/kg/day. Coadministration of suboptimal doses of tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) prolonged skin graft rejections dramatically (55% of the grafts survived for more than 40 days, n=9). Taken together, these results demonstrate that tepoxalin is a potent immunomodulatory compound that, when combined with CsA, provides synergistic immunosuppressive activity. The fact that tepoxalin and CsA act on different transcription factors, NFκB and NFAT respectively, might explain the synergistic suppressive effects when both compounds were used. Tepoxalin could be an important addition to the cohort of immunosuppressive therapies currently used in solid organ and bone marrow transplantations.

The immune response in transplantation, which results in graft rejection and graft-versus-host (GVH)[a] response, is primarily triggered by T cells through recognition of alloantigens (1–4). Suppression of immune response could be achieved using agents interfering with T cell activation and effector functions. The use of cyclosporine (CsA) as an immunosuppressant in transplantation has been documented (5, 6). CsA inhibits T cell activation by inhibiting the nuclear translocation of the nuclear factor NFAT (7, 8). However, CsA has associated toxicities and side effects when used at

therapeutic doses (9). Compounds that suppress T cell-mediated immune response with mechanisms different from that of CsA will undoubtedly be valuable additions to the cohort of the current regiments.

Tepoxalin (5-[4-chlorophenyl]-N-hydroxy-[4-methoxyphenyl]-N-methyl-1-H-pyrazole-3-propanamide) was discovered originally as a dual inhibitor of 5-lipoxygenase (LO) and cyclooxygenase (CO) and exhibits potent nonsteroidal antiinflammatory activities in animal models of adjuvant arthritis (10–12). Recently we found that tepoxalin also inhibits OKT3-induced T cell proliferation via a mechanism very different from that of CsA (13). CsA is known to block IL-2 production after activation of T cells through TCR/CD3, whereas tepoxalin inhibits IL-2 induced signal transduction (13). An in-depth investigation of the mechanism of action reveals that tepoxalin inhibits predominantly NFκB activation (14), whereas CsA is most effective in blocking NFAT transactivation (7, 8). Because of these different mechanism of actions, a possible additive/synergistic effect of the combined tepoxalin and CsA treatment is expected. In this report, we demonstrate that tepoxalin is indeed effective in suppressing mixed lymphocyte reactions (MLR), GVH responses, and allogeneic skin graft rejections in mice. The synergistic effect of tepoxalin and CsA in immunosuppression was also studied. The possible mechanism of tepoxalin in immunosuppression and its potential clinical application are discussed.

MATERIALS AND METHODS

Mice. Inbred C57BL/6J, C3H/HeJ, and BALB/cByJ mice and B6D2F₁/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Those used in experiments were male mice at about 6–10 weeks of age that weighed 18–25 gs.

Preparation of test compounds. Tepoxalin, naproxen, and zileuton were synthesized by the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). CsA (Sandimmune i.v.) was from Sandoz (Quebec, Canada). For MLR experiments, stock solutions of tepoxalin, naproxen, and zileuton were prepared in DMSO at 30 mM and diluted to working concentrations in culture medium at the time of experiments. DMSO at concentrations equivalent to those of the test compounds were used as controls in MLR assays. For experiments of GVH responses and skin graft rejections, micronized tepoxalin and naproxen were suspended in 0.5% methylcellulose (Sigma, St. Louis, MO) at concentrations of 5 mg/ml or lower. The vehicle control was the equivalent volume of 0.5% methylcellulose. Zileuton was dissolved in 50% polyethylene glycol 200 (Sigma, St. Louis, MO) and the corresponding vehicle control was the equivalent volume of polyethylene glycol 200. CsA was diluted in saline. All compounds were dissolved in vehicle just prior to administration to mice at volumes of

[a] Abbreviations: CsA, cyclosporine; CO, cyclooxygenase; GVH, graft-versus-host; LO, 5-lipoxygenase; MLR, mixed lymphocyte re-

Lymphocyte proliferation assays. Single-cell suspensions from mouse spleens were washed once with PBS and then resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μM 2-mercaptoethanol. Responder spleen cells (2.5×10^7) from C57BL/6J mice ($H-2^b$) were stimulated by 2.5×10^6 irradiated (2000 rads) spleen cells from B6D2F₁/J mice ($H-2^{b/d}$). The responder and the stimulator cells were cocultured in 250 μl medium containing various concentrations of the tested compounds in the 96-well plates (round bottom wells, Corning Inc., NY). After 5 days of stimulation, ³H-thymidine was added to the cultures (0.5 μCi per well) for 4 hr. Plates were harvested using a Tomtec Harvester 96, MACH II (Tomtec Inc., Orange, CT) and samples were counted using a Wallac 1205 Betaplate scintillation counter (Pharmacia, Uppsala, Sweden).

Cell viability test. Cell viability was assessed with the MTT assay. Spleen cells from C57BL/6J mice were prepared in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 μM 2-mercaptoethanol. Spleen cells (2×10^5/well) were stimulated with immobilized anti-CD3 (Pharmingen) in the presence of tepoxalin or its vehicle DMSO in 96-well culture plates (Corning Inc., NY). The MTT assay was conducted by using the Celltiter 96 kit (Promega Corp.), which is based on the conversion of a tetrazolium salt by viable cells into a detectable blue formazan.

Graft-versus-host reactions. The GVH assay was based on the method of Dorsch and Roser (15). Spleen cells from C57BL/6J mice were injected subcutaneously into the footpads of B6D2F₁/J mice. Each footpad was injected with 8×10^6 spleen cells in 50 μl. Seven days later, the draining popliteal lymph nodes were removed, trimmed off fat and weighed. Mice injected in the footpads with saline were used as negative controls. Lymph nodes of these mice were indistinguishable from those injected with syngeneic spleen cells. Tepoxalin was administered orally and CsA was given subcutaneously to mice daily started one day before footpad injection unless otherwise specified.

Skin graft transplantation. C3H/HeJ mice ($H-2^k$) were anesthetized by intraperitoneal injection of 2.5% avertin (0.016 ml/g body weight). A grafting bed (about $0.3 \text{ cm} \times 1$ cm) on the mouse tail was prepared by peeling off skin carefully to avoid bleeding. Tail skin of similar size was peeled from BALB/cByJ mice ($H-2^d$) and then placed over the graft site in an opposite orientation according to the hair growth direction. The grafted skin was protected by a plastic tubing (diameter 0.5 cm, length 3 cm) held in place by wound clips for 5 days. Skin grafts were examined and scored daily. A graft was scored as being rejected when more than 80% of the graft was necrotic. CsA was given subcutaneously to mice daily starting one day before skin transplantation until rejection of grafts. Tepoxalin was given orally one day before transplantation and then daily starting one day after transplantation until graft rejection.

Data presentation and statistics. Data were analyzed using one-tailed Dunnett's tests. A parametric version was used if data were normally distributed as assessed by the Wilk-Shapiro test. Data which did not meet the assumptions of normality were tested using a nonparametric version of the Dunnett's test.

RESULTS

Inhibition of MLR proliferations by tepoxalin. We recently reported that tepoxalin suppresses T cell proliferation and inhibits the activity of the transcription factor NFκB (13, 14). T cell activation and proliferation are critical for the initiation of an antigen specific immune response. The transcription factor NFκB is also known to be involved in regulating the expression of many target genes in an immune response (16, 17). The possible immunosuppressive effect of tepoxalin was therefore studied. To determine whether tepoxalin is capable of inhibiting the immune response against alloantigens, tepoxalin at various concentrations was tested in MLR proliferation assays. The assay was set up by stimulating

C57BL/6J ($H-2^b$) mouse spleen cells with irradiated B6D2F₁/J ($H-2^{b/d}$) mouse spleen cells. As shown in Figure 1, tepoxalin inhibited cell proliferation in a dose-dependent fashion with an IC_{50} of 1.3 μM. The inhibitory effect was not related to cell toxicity. Tepoxalin at concentrations of 25 μM or less did not affect the viability of anti-CD3 stimulated mouse spleen cells after 24 hr of treatment (Table 1). Since tepoxalin is a dual CO/LO inhibitor (10), the possible link of its suppression of MLR proliferation to its inhibition of CO and/or LO was studied. To address this question, the well-known CO inhibitor naproxen and the LO inhibitor zileuton were tested in parallel at doses 10-fold higher than their IC_{50} for suppression of CO or LO in mice, respectively. Neither of these compounds, nor the combination of both of them, had an inhibitory effect on MLR proliferation (Fig. 1).

To further understand the mechanism of action of tepoxalin, the kinetics of tepoxalin in inhibiting MLR proliferation was compared to that of the known immunosuppressant, CsA. As shown in Table 2, the inhibitory effect was not diminished when tepoxalin was added 24–72 hr after the initiation of MLR. In contrast, CsA was effective only if it was added at the beginning of the cocultures. To determine whether tepoxalin and CsA were synergistic in inhibiting MLR proliferation, the two agents were tested in combination. Tepoxalin at 0.5 μM, 1 μM, or 2 μM was tested in combination with varying concentrations of CsA (Fig. 2). CsA alone inhibited the response in a dose-related manner with an IC_{50} of 22 nM. Tepoxalin alone inhibited proliferation by 26% at 0.5 μM, by 55% at 1 μM, and by 87% at 2 μM. When tepoxalin and CsA were present at suboptimal concentrations, the inhibition was clearly additive. This additive effect was less significant at concentrations of the two drugs that were strongly inhibitory on their own.

Suppression of GVH responses by tepoxalin. The immunosuppressive effect of tepoxalin as demonstrated in MLR as-
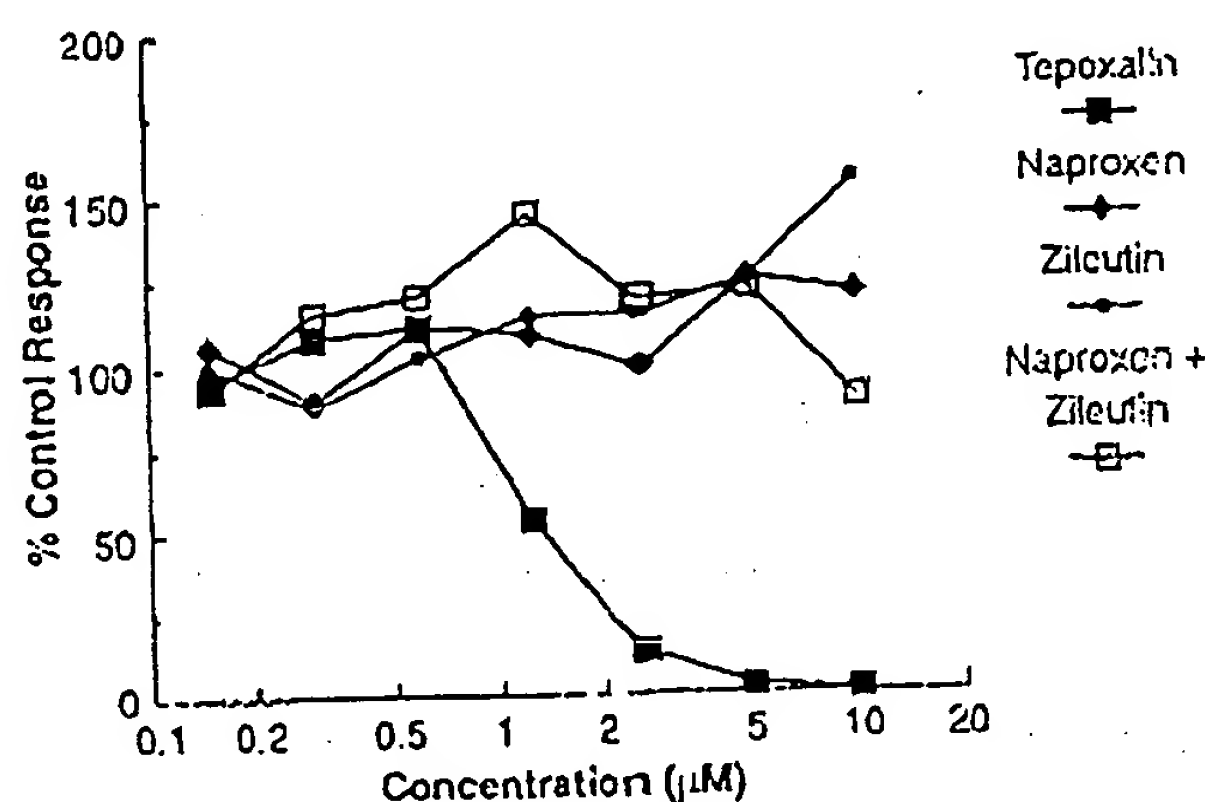

FIGURE 1. Inhibition of MLR proliferations by tepoxalin. Spleen cells from C57BL/6J mice were cocultured in triplicate wells with irradiated spleen cells from B6D2F₁/J mice as described in *Materials and Methods*. Varying concentrations of tepoxalin, naproxen, zileuton, or naproxen + zileuton were added to the cultures at the initiation of cultures. ³H-thymidine uptake was measured on day 5. Control cultures contained DMSO diluted in a manner similar to that of the compounds. Uptake of ³H-thymidine in vehicle controls was about 90,000 cpm. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

TABLE 1. The effective doses of tepoxalin in immunosuppression is not toxic to cells[a]

Tepoxalin (μM)	Cell viability[b]
100	52.0%
50	76.4%
25	96.5%
12.5	101.0%
6.25	115.5%
3.12	118.4%
1.56	136.5%

[a] Viability of anti-CD3 stimulated C57BL/GJ spleen cells treated with tepoxalin for 24 hr was tested in the MTT viability assay.

[b] Cell viability is presented as the percentage of viable cells in tepozalin treated sample compared with that treated with an equivalent amount of the vehicle, DMSO.

TABLE 2. Inhibitory effect of tepoxalin and CsA on MLR proliferations (% control response)[*]

	Concentration (μM)	Time of Treatment			
		0 hr	24 hr	48 hr	72 hr
Tepoxalin	1.25	64.0	52.6	67.0	30.6
	2.5	17.2	12.7	19.2	15.1
	5.0	4.7	5.2	7.8	9.0
Cyclosporine	0.021	40.6	65.0	97.1	133.6
	0.042	18.6	99.5	93.2	148.0
	0.084	5.7	61.1	84.4	119.2

[*] Different concentrations of compounds added in MLR cultures at different time points were studied. MLR assays were set up as described in *Materials and Methods*. The MLR proliferations treated with compounds were compared with their vehicle controls. [3]H-thymidine uptake by proliferating cells in MLR assays was measured. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

says suggests its potential use as an immunosuppressant in clinical therapy. This possible application was verified with *in vivo* murine models of transplantation. A local GVH response was performed by injecting spleen cells from the parental C57BL/GJ (H-2[h]) mice into the footpads of B6D2F1/J (H-2[Md]) mice. GVH responses were demonstrated by the enlargement of the draining popliteal lymph nodes in recipient mice. The lymph nodes of recipient mice increased significantly by day 2 and continued to increase in size with time. The degree of the local GVH response was measured by weighing the draining popliteal lymph nodes. The lymph nodes of tepoxalin-treated mice did enlarge on day 2 but did not change significantly later on. After 7 days of the local GVH response, lymph nodes from tepoxalin-treated mice were slightly hyperplastic, but were significantly less so than that of the untreated controls (Fig. 3A). GVH responses in mice administered tepoxalin orally at 12–50 mg/kg/day were reduced by about 40% of that in the positive control group. Consistent with the findings in mice, tepoxalin was also effective in rats, with a 30% suppression of this local GVH response at 12 mg/kg/day (data not shown). The immunosuppressive agent CsA administered subcutaneously to mice at 50 and 75 mg/kg/day was shown to suppress GVH response by 42% and 71%, respectively (Fig. 3B). The results suggest that the immunosuppressive effect of tepoxalin at 12 mg/kg/day is comparable to that of CsA at 50 mg/kg/day. To assess whether the inhibitory effect of tepoxalin on GVH responses
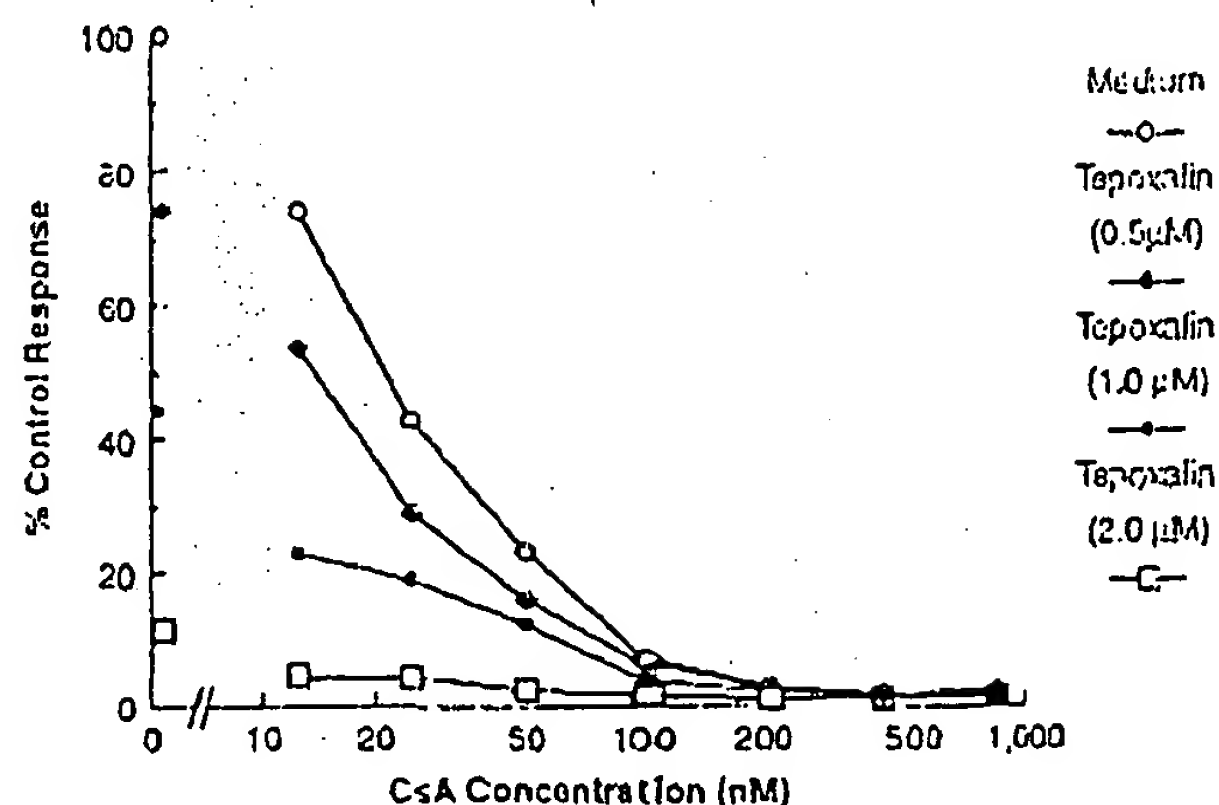
FIGURE 2. Additive inhibitory effects of tepoxalin and CsA in MLR proliferations. Proliferation of C57BL/GJ mouse spleen cells after 5 days stimulation with irradiated B6D2F1 spleen cells in medium containing tepoxalin at 0.5 μM, 1 μM, and 2 μM plus varying concentrations of CsA was assayed as described in *Materials and Methods*. The proliferative response in cultures containing no drugs was 80,000 cpm.

could be obtained with other CO or LO inhibitors, naproxen and zileuton were again tested in GVH assays. No inhibition was seen with zileuton, naproxen, or a combination of the two compounds (Fig. 3C).

Since tepoxalin appears to act late in MLR assays, the effect of tepoxalin administered early and late in GVH responses was also studied. Similar to the findings in MLR proliferations, tepoxalin given to mice for a minimum of 3 days was sufficient to suppress GVH responses to an extent similar to those treated with tepoxalin throughout the 7-day course of the GVH response (Fig. 4). This short treatment with tepoxalin could be at the early (day −1 to day 1 or 4) or the late (day 4 to day 6) stage of the GVH response. The inhibitory effect of tepoxalin at the late stage of immune responses suggests its mechanism of action to be different from that of CsA. The possible synergism in immunosuppression by tepoxalin and CsA was therefore studied in GVH assays. A much stronger suppression of the GVH response was indeed found in mice treated with both tepoxalin and CsA rather than those treated with either one of the two drugs (Fig. 5). This synergistic effect was particularly significant when a low dose of tepoxalin (6 mg/kg/day) was combined with CsA.

Prolongation of skin allograft survival by tepoxalin. The time course of skin allograft rejection in mice is affected by the efficiency of the following two mechanisms: (1) the activation of T cells through recognition of specific alloantigens, and (2) the effector mechanisms mediating tissue distruction. To study the effect of tepoxalin on skin allograft survival, experimental allograft rejection was performed by grafting allogeneic BALB/cByJ (H-2[d]) mouse tail-skin onto C3H/HeJ (H-2[k]) recipient mice. For the first 6 days after transplantation, allografts appeared normal and their gross appearance was not different from that of syngeneic grafts. The rejection process became apparent by day 6, with signs of swelling and erythema, and quickly culminated into complete graft necrosis. Different doses of tepoxalin were tested in skin graft rejection assays. As shown in Figure 6, rejection of allografts
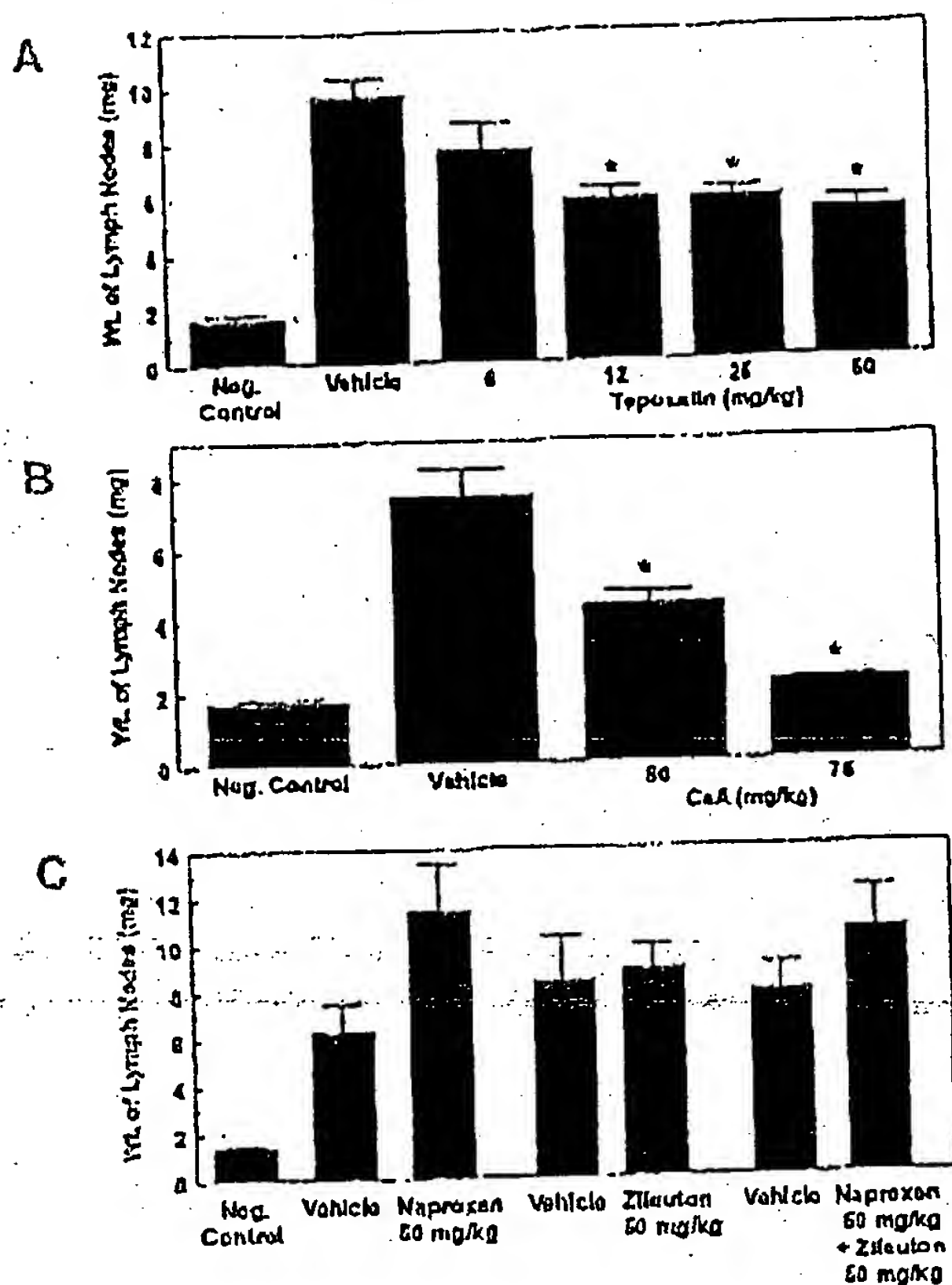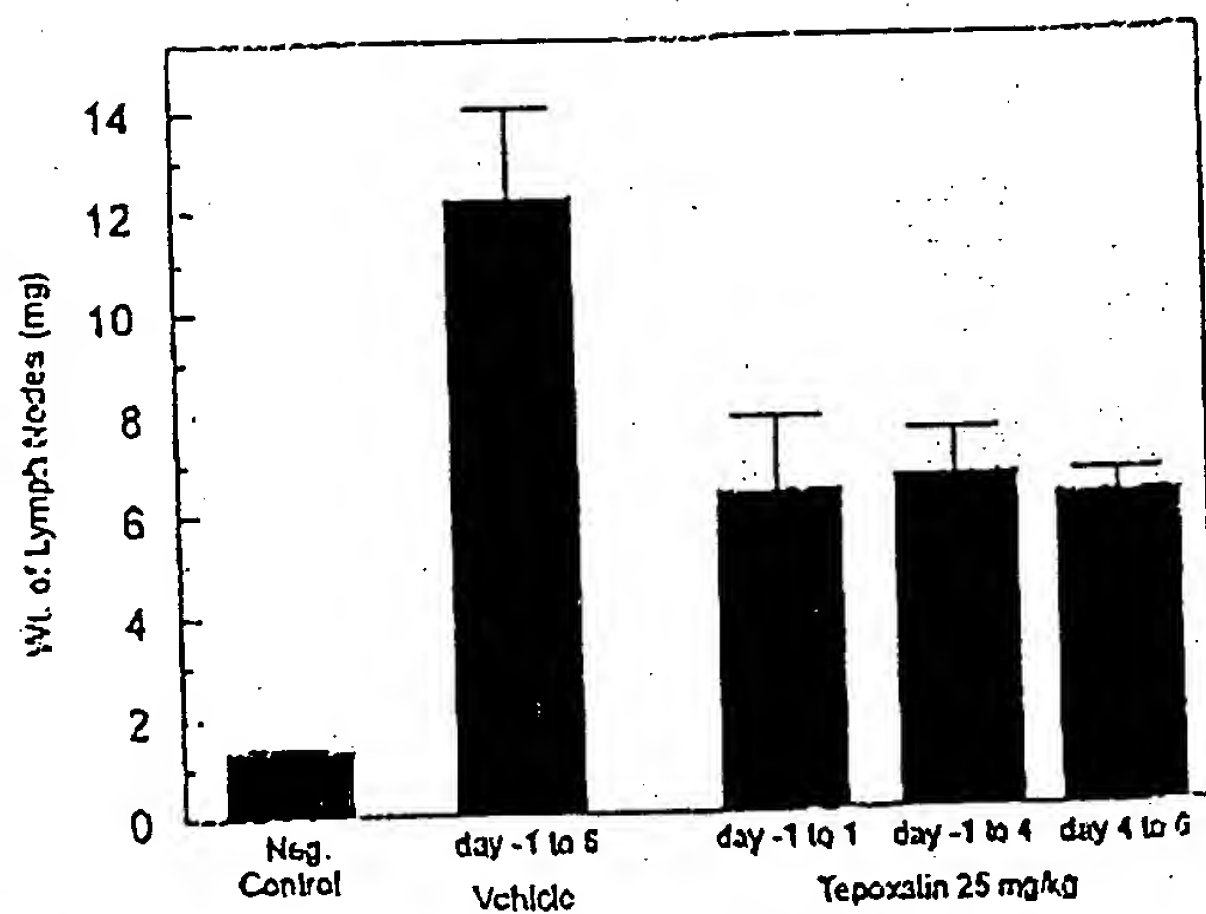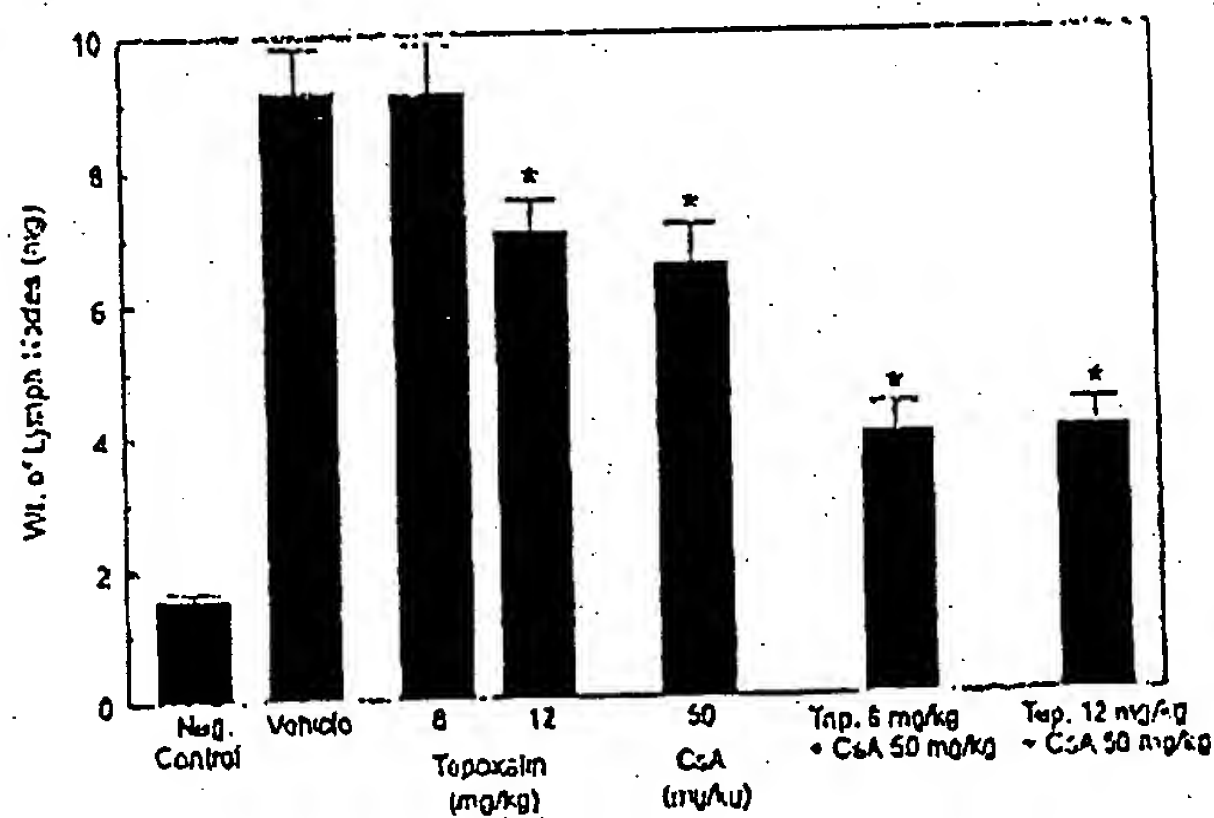
FIGURE 3. Suppression of GVH responses by tepoxalin. A local GVH response was triggered by subcutaneous injection of parental C57Bl/6J spleen cells into footpads of B6D2F₁/J mice, and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice injected with saline were used as negative controls. Drugs were given to mice from day −1 to day 6 of the GVH response. (A) GVH responses in mice administered different doses of tepoxalin or vehicle control (0.5% methylcellulose) orally. Ten mice were used per group. The values from mice treated with tepoxalin at 12, 25, and 50 mg/kg/day are significantly different from the vehicle control group (Dunnett's test). Similar results were obtained from more than three repeated experiments. (B) GVH responses in mice given CsA (50 and 75 mg/kg) or vehicle control (saline) subcutaneously. Five mice were used per group. (C) GVH responses in mice given naproxen, zileuton, or the combination of the two drugs at 50 mg/kg/day orally. Mice as vehicle controls for naproxen were treated with equivalent volumes of 0.5% methylcellulose; for zileuton, they were treated with 50% polyethylene glycol 200; and for the combination of drugs, they were treated with both 0.5% methylcellulose and 50% polyethylene glycol 200. Five mice were used per group. The column bars represent the standard errors. Asterisks indicate a *P* value of <0.05.

in the placebo-treated group started on day 7. About 50% of the allografts in the placebo group were rejected on day 10. Tepoxalin at doses of 12.5 and 25 mg/kg/day did not have a significant effect in prolonging graft rejection. When tepoxalin at 50 mg/kg/day was administered to mice, a significant prolongation of skin graft rejection was observed. The median survival time of skin grafts, defined as the time point at which 50% of the grafts are rejected, was 10.5 days in the

FIGURE 4. Effective suppression of mouse GVH response by short treatments with tepoxalin. The GVH response was induced by injection of C57Bl/6J spleen cells into the footpads of B6D2F₁/J mice and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice injected with saline instead of spleen cells were used as negative controls. Tepoxalin (25 mg/kg) was administered orally to mice at different time schedules as shown. GVH responses in mice treated with vehicle (0.5% methylcellulose) were used as positive controls. Five mice were used per group. The column bars represent the standard errors.

FIGURE 5. Synergistic suppression of mouse GVH responses by tepoxalin and CsA. The GVH response was induced by injection of C57Bl/6J spleen cells into the footpads of B6D2F₁/J mice and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice were treated with CsA (50 mg/kg) or tepoxalin (6 or 12 mg/kg) alone, or the combination of tepoxalin (6 or 12 mg/kg) and CsA (50 mg/kg). Mice injected with spleen cells and treated with vehicles were used as positive controls. Mice injected with saline instead of spleen cells were used as negative controls. Twenty mice were used per group. The column bars represent the standard errors. Asterisks indicate a *P* value of <0.05. Similar results were obtained from repeated experiments.

placebo-treated group and was 15.0 days for the group of mice treated with tepoxalin at 50 mg/kg/day (*P*<0.05). Furthermore, a combination of tepoxalin and CsA at low doses showed a dramatic prolongation of allogeneic skin graft rejection (Fig. 7). About 52% of the mice treated daily with
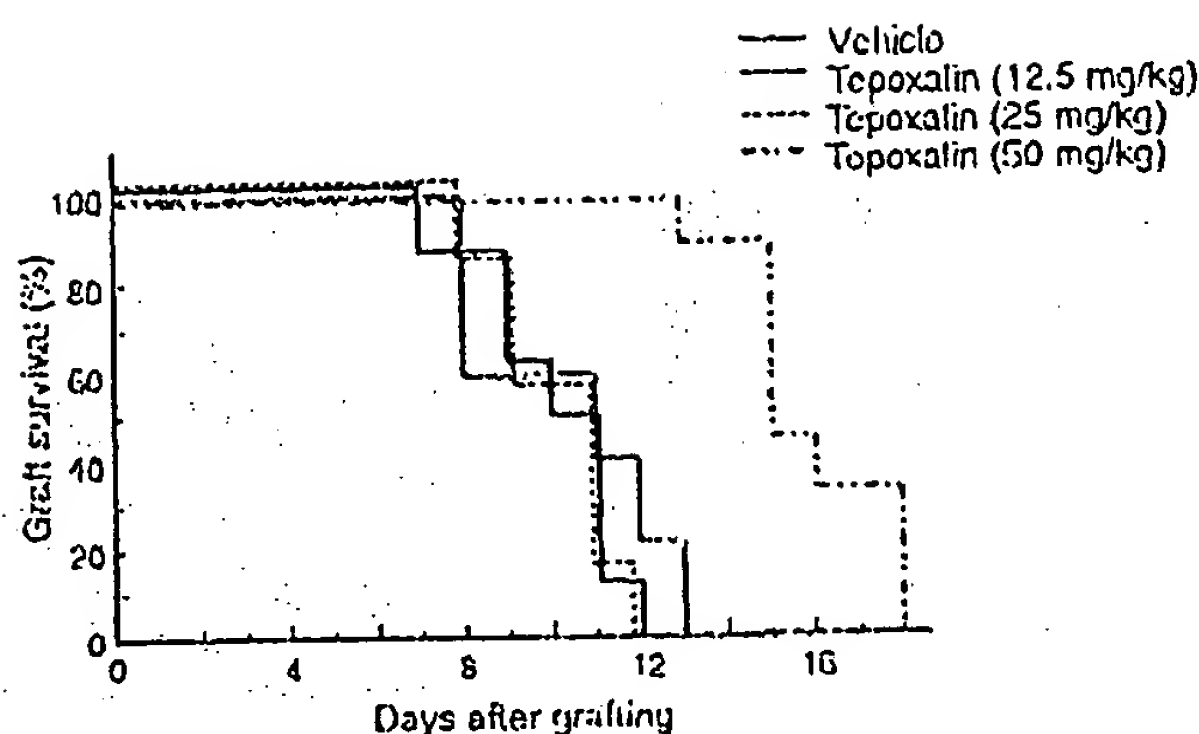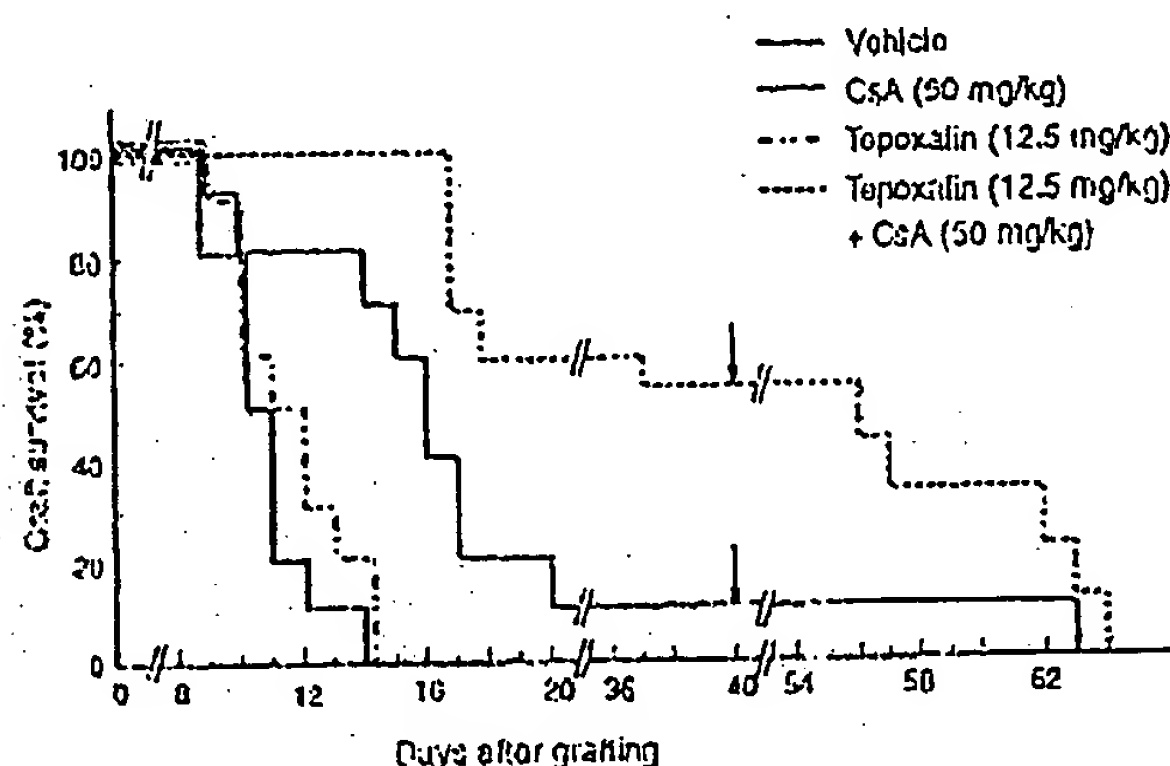
FIGURE 6. Prolongation of skin graft rejections by tepoxalin. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice and rejection of the grafted skin was scored as described in *Materials and Methods*. Different doses of tepoxalin were administered orally to C3H/HeJ recipient mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Mice given the vehicle (0.5% methylcellulose) orally were used as controls. About ten mice were used per group. Data presented were taken from one of the three repeated experiments. Results obtained from all three experiments were similar. Prolongation of skin rejection in mice treated with tepoxalin 50 mg/kg was significant ($P<0.05$, Dunnett's *t* test).

FIGURE 7. Enhanced prolongation of allogeneic skin graft rejection by tepoxalin and CsA. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice as described in *Materials and Methods*. Tepoxalin (12.5 mg/kg) alone, CsA (50 mg/kg) alone, or tepoxalin (12.5 mg/kg) plus CsA (50 mg/kg) were administered to C3H/HeJ mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Tepoxalin was given orally and CsA was given subcutaneously. For recipient mice with skin grafts surviving for more than 40 days, drug administration was discontinued from day 40, as shown by arrows. About ten mice were used per group. Enhanced prolongation of skin rejection was also observed for the combination of tepoxalin (25 mg/kg) and CsA (50 mg/kg) (data not shown).

tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) retained the allogeneic skin grafts on day 40 after transplantation. To determine whether immunotolerance to skin grafts is generated by the combined drug treatment, drug dosing was discontinued after day 40 of transplantation. Skin graft rejection was noticeable on day 16 and all the grafts were rejected

on day 24 after drug cessation (Fig. 7). The results suggest that the combination of tepoxalin and CsA potentiates the immunosuppressive effect, but does not induce immunotolerance to the grafts.

DISCUSSION

In this report, we demonstrate that tepoxalin is effective in suppressing the immune responses in murine models of GVH reaction and allogeneic skin graft rejection. This immunosuppressive activity is not seen with other inhibitors of CO or LO.

To study the mechanism of immunosuppression by tepoxalin, we used the *in vitro* mixed lymphocyte reaction, which measures the proliferative response of parental strain C57BL/6J spleen cells when stimulated by B6D2F$_1$/J spleen cells. Tepoxalin inhibited the alloantigen-driven proliferative response in a dose-related manner with an IC_{50} of 1.3 µM and a complete inhibition at 5 µM. A similar inhibition was seen with CsA, which had an IC_{50} of approximately 22 nM and a complete inhibition at about 200 nM. However, there were differences in the kinetics of the inhibitions seen with the two compounds. Tepoxalin exerted the same degree of inhibition if added any time up to 72 hr after the set-up of MLR cultures. CsA was only inhibitory if added at the initiation of the MLR cultures. IL-2 production by T cells occurs early following activation, reaching peak levels by 24 hr of culture (18, 19). CsA has been known for its inhibitory effect on IL-2 production (7, 20, 21) and is therefore expected to affect T cells during the first 24 hr of activation. The fact that tepoxalin inhibits proliferation late in MLR assays suggests its inhibition of later events in T cell activation. One possibility is that the IL-2-mediated signal transduction pathway is affected by tepoxalin, which has been shown on human lymphocytes in our previous report (13).

GVH disease is a common problem in bone marrow transplantation that leads to frequent morbidity and mortality (22). Skin grafts trigger strong immune responses and have been one of the most difficult grafts in transplantation (3). The immunosuppressive activity of tepoxalin was demonstrated in murine models of GVH responses and allogeneic skin graft rejections. Tepoxalin was found to inhibit GVH responses at 12 mg/kg/day and to prolong skin graft rejections at 50 mg/kg/day. The possibility that tepoxalin blocks a later event in immune response is again implicated by its suppression of GVH reaction even when it was administered to mice 4 days after the initiation of the response.

Tepoxalin is known to be a dual CO and LO inhibitor with potent antiinflammatory effects (10). One of the obvious questions to ask is whether its immunosuppression is due to the inhibition of the CO or LO enzymes. The involvement of CO and LO in the modulation of immune responses remains controversial. Arachidonic acid metabolites produced by these enzymes, such as prostaglandins and leukotrienes, have many biological activities, including the modulation of inflammation and immune response (23–29). Indeed several inhibitors of LO have been shown to prolong graft rejection in transplantation (30–33). However, it was noticed that those LO inhibitors with immunosuppression activity are also potent antioxidants with inhibitory effects on NFκB activity (34, 35). Therefore the immunoregulatory effects of these

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compounds may not be directly related to inhibition of LO. We compared the effect of tepoxalin with other known LO/CO inhibitors in our studies. Naproxen (CO inhibitor) or zileuton (LO inhibitor), or their combination, did not have any effect on MLR proliferations or GVH responses. We have reported recently that tepoxalin is distinct from other CO and LO inhibitors in its inhibition of NFκB activities (14). NFκB is a pleiotropic transactivator of many target genes involved in immune or inflammatory responses (16, 17). The immunosuppressive effect of tepoxalin may be attributed to its inhibition of NFκB and not related to the general inhibition of arachidonic acid metabolism.

Taken together, these data show that tepoxalin is an effective immunosuppressive agent. Since the mechanism of tepoxalin appears to be different from CsA in immunosuppression, it suggests a possible combinational use of the two compounds in immunosuppressive therapy. Moreover, tepoxalin is devoid of ulcerogenic actions in gastrointestinal systems that are the common side effects of other NSAID drugs (11, 12). The LD_{50} of tepoxalin in mice and rats was more than 400 mg/kg, which is over 10-fold higher than the effective doses used in *in vivo* immunosuppression. Tepoxalin could therefore be an important addition to the existing immunosuppressive therapeutic drugs to enhance the efficacy of treatment and to reduce drug toxicity in transplantation and autoimmunity.

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COMBINED THERAPY WITH INTERLEUKIN-4 AND INTERLEUKIN-10 INHIBITS AUTOIMMUNE DIABETES RECURRENCE IN SYNGENEIC ISLET-TRANSPLANTED NONOBESE DIABETIC MICE

ANALYSIS OF CYTOKINE mRNA EXPRESSION IN THE GRAFT[1]

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Syngeneic pancreatic islet grafts in nonobese diabetic (NOD) mice elicit a cell-mediated autoimmune response that destroys the insulin-producing β cells in the islet graft. IL-4 and IL-10 are cytokines that inhibit cell-mediated immunity. In this study, we evaluated the effects of IL-4 and IL-10 on the survival of syngeneic pancreatic islets transplanted into diabetic NOD mice. Islet grafts survived beyond 18 days and normoglycemia was maintained in 67% (10 of 15) of mice treated with IL-4 plus IL-10, but in none (0 of 20) of vehicle-injected (control) mice. Also, 40% (6 of 15) of the mice treated with IL-4 plus IL-10 were normoglycemic at 90 days after transplantation, compared with 14% (1 of 7) of the mice treated with IL-4 alone, 8% (1 of 13) of the mice treated with IL-10 alone, and none (0 of 20) of the control mice. Histological examination of grafts at 10 days after transplantation revealed periislet accumulations of mononuclear leukocytes and intact islet β cells in grafts from IL-4 plus IL-10-treated mice, whereas islets were infiltrated by leukocytes and the β cell mass was greatly reduced in grafts from control mice. Polymerase chain reaction (PCR) analysis of cytokine mRNA expression in the grafts revealed higher levels of IL-2, IFNγ, and IL-10 mRNA in grafts of diabetic compared with normoglycemic control mice, whereas IFNγ and TNFα mRNA levels were significantly decreased in grafts of IL-4 plus IL-10-treated mice compared with either normoglycemic or diabetic control mice. These results suggest that T helper (Th)1 cells and their cytokine products (IL-2, IFNγ, and TNFα) may promote islet β cell destructive insulitis and autoimmune diabetes recurrence in syngeneic islet-transplanted NOD mice, and that administration of IL-4 plus IL-10 may inhibit diabetes recurrence by suppressing Th1 cytokine production in the islet grafts.

Insulin-dependent diabetes mellitus (IDDM)* results from destruction of the insulin-producing pancreatic islet β cells by the host's own immune system. Whereas it is not known what may initiate this autoimmune response against islet β cells, there is abundant evidence that IDDM is T cell-dependent (1, 2). However, it is unclear which T cells are involved and how they may lead to islet β cell destruction. A variety of immune/inflammatory cells infiltrate the pancreatic islets and constitute the insulitis lesion (3, 4). There is evidence in human patients with IDDM (5–8) and in animals with spontaneous IDDM resembling the human disease—the nonobese diabetic (NOD) mouse and the biobreeding (BB) rat (9–22)—that islet β cell destruction may involve heterogeneous effec-

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* Abbreviations: BB, Biobreeding; CFA, complete Freund's adjuvant; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; PCR, polymerase chain reaction; Th, T helper.

Immunomodulation by an Adenylate Cyclase Activator, NKH477, in Vivo and in Vitro

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Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger which modulates T cell function. NKH477 is a direct adenylate cyclase activator derived from forskolin and now under clinical investigation as a positive inotropic agent. While the immunosuppressive effects of forskolin on lymphocytes have been reported, little is known about its effects *in vivo*. In this study, we investigated whether NKH477 has immunosuppressive effects in mice, namely on cardiac allograft survival, and on the generation of cytotoxic T lymphocytes (CTL), T cell proliferation in mixed lymphocyte reaction (MLR), and production of interleukin-2 (IL-2) in MLR and in mitogen response. We assessed the effects of standard immunosuppressant cyclosporin A (CsA) on IL-2 production and on allograft survival to estimate the intensity of rejection in this acute rejection model. Saline-treated C57BL/6 (H-2^b) mice rejected DBA/2 (H-2^d) cardiac allografts with a median graft survival time of 10 days. In contrast, median graft survival was prolonged to 12 and 15 days in mice treated with NKH477 at 1 and 3 mg/kg/day, respectively (*P* < 0.01 vs control). The equivalent dose of CsA (40 mg/kg/day) to the maintenance dose after clinical cardiac transplantation prolonged median graft survival time to 15.5 days, indicating that high dose of NKH477 was as efficacious as lower dose of CsA. Addition of NKH477 to the culture medium suppressed the generation of CTL, T cell proliferation in MLR, and production of IL-2 in MLR and in mitogen response. These results suggest that NKH477 exerts a beneficial effect on murine cardiac allograft survival by modulating T cell function. © 1996 Academic Press Inc.

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger which has various modulatory effects on the immune system. Forskolin, prostaglandin E$_2$, and other cAMP-elevating agents have been reported to have immunosuppressive effects such as decreased production of interleukin-2 (IL-2) (1, 2). An increase in [cAMP]$_i$ is also considered to have an inhibitory effect on the generation of cytotoxic T lym-

phocytes (CTL) (3–6), which are considered playing a role in the course of acute allograft rejection (7–10). IL-2 is involved both in the proliferation of mature T cells and in the generation of CTL (11–17).

Forskolin directly activates adenylate cyclase and has been routinely used in *in vitro* studies investigating the effect of increased [cAMP]$_i$. However, on the grounds of the low solubility of this compound in water and its poor biological availability on oral administration, it has been unclear whether increased [cAMP]$_i$ by adenylate cyclase activators has an immunosuppressive effect *in vivo*.

NKH477, (+)-(3R,4aR,5S,6S,6aS,10S,10aR,10bS)-5-acetoxy-6-(3-dimethylaminopropionyloxy)-dodeca hydro-10,10b-dihydroxy-3,4a,7,7,10a-pentamethyl-3-vinyl-1H-naphtho-[2,1-b]pyran-1-one monohydrochloride, is a new water-soluble forskolin derivative now under clinical investigation as a positive inotropic agent. Like forskolin, NKH477 directly activates the catalytic unit of adenylate cyclase and increases [cAMP]$_i$ (18, 19). It has been shown to have beneficial effects on hemodynamic states, especially on diastolic function, in an experimental model of congestive heart failure (20). The drug also has the unique characteristic of suppressing both digitalis- and adrenaline-induced ventricular arrhythmia, in contrast to phosphodiesterase inhibitors, and can be used safely with little arrhythmogenicity (21). These characteristics may be beneficial in the treatment of heart failure developing as a manifestation of acute allograft rejection.

In this study, we investigated the effects of NKH477 on cardiac allograft survival in a murine heterotopic cardiac transplantation model. We also used cyclosporin A (CsA) as a standard immunosuppressant to estimate the intensity of rejection in this acute rejection model. One-way MLR was performed to evaluate antigen-specific CTL generation and IL-2 production in consideration of the results of measurement of plasma NKH477 and M1 (equally active metabolite of NKH477) concentration. The immunosuppressive effects of NKH477 *in vitro* were compared to CsA for the inhibition of IL-2 production in MLR and in mitogen

response, since the effects of CsA are manifested mostly through suppression of IL-2 production. We also assessed the proliferative response of splenic T cells during one-way MLR and to exogenous IL-2 by [^{3}H]thymidine incorporation.

METHODS

Mice

Male DBA/2 (H-2^d), C57BL/6 (H-2^b), and C3H/He (H-2^k) mice aged 7–9 weeks were obtained from the Shizuoka Agricultural Cooperation Association (Shizuoka, Japan), housed in stainless-steel cages with controlled 12-hr light/dark cycle and given access to standard mouse chow and water.

Reagents

NKH477 (MW 546.1) was provided as a pure powder by Nippon Kayaku Co., Ltd. (Tokyo, Japan). It was dissolved in saline and adjusted to pH 4 with 0.01 N HCl. Solutions were stored at 4°C. Orally available CsA (Sandimmun drink solution; Sandoz Corporation, Basel, Switzerland) was commercially obtained and diluted with olive oil. CsA for *in vitro* use was a gift from Sandoz Corp. Stock solutions of CsA were prepared at a concentration of 30 mg/kg in dimethyl sulfoxide and diluted to the required concentrations with culture medium. In *in vivo* experiments, NKH477 was given at 0.3, 1, or 3 mg/kg/day and CsA was at 5, 10, or 40 mg/kg/day, by single daily oral administration beginning on the day of transplantation. RPMI 1640, FCS, and HBSS (Hanks' balanced salt solution) were purchased from Gibco Laboratories (Grand Island, NY). Mitomycin C and concanavalin A (Con A) were obtained from Sigma Chemical Co. (St. Louis, MO). LPS from *Esherichia coli* 055:B5 was from Difco (Detroit, MI). Recombinant human IL-2 and [*methyl-*^{3}H]-thymidine were from Amersham (Buckinghamshire, UK). Anti-Thy-1.2 monoclonal antibody and low toxic rabbit serum complement for cytotoxic elimination test were from Cedarlane Laboratories (Hornby, Ontario, Canada).

Cardiac Transplantation

DBA/2 mice served as transplant donors and C57BL/6 mice as recipients. Heterotopic cardiac transplantation was performed as previously described (22, 23). In brief, donors and recipients were anesthetized with 4% chloral hydrate at 0.01 ml/g body weight ip prior to surgery. Donor hearts were perfused with heparinized saline chilled to 4°C via the inferior vena cava and harvested after ligation of the vena cavas and pulmonary veins. The donor ascending aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava using a microsurgical technique. Success rate was approximately 85%. Graft

failures arising within the first 3 days after transplantation were excluded from experiments as technical failures. Grafts were monitored by daily abdominal palpation and regular electrocardiogram. Rejection was defined as the day of cessation of heartbeat.

Histopathological Examination

Recipients were killed 5 days after transplantation by bleeding after being anesthetized with ether. Allografts were harvested, sectioned transversely at the maximal circumference of the ventricle, and fixed in 10% buffered formalin. The graft tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathological examination was conducted by two observers who were unaware of any background data. The severity of myocardial inflammation, perivascular inflammation, and necrosis was independently graded for severity by two blinded observers on a scale of 1 (low grade), 2 (moderate grade), and 3 (high grade), and scores were averaged.

Measurement of Plasma NKH477 and M1 Concentration

Seven-week-old C57BL/6 mice were orally administered NKH477 at a single daily dose 3 mg/kg for 5 days. They were anesthetized by inhalation of ether at 30 min or 1, 2, 4, 8, or 24 hr after last administration. Blood was collected by cannulation of the inferior vena cava, heparinized, and centrifuged. Collected plasma were frozen at −70°C until measurement of the concentration of NKH477 and M1 concentrations by gas chromatography/mass spectrometry. M1 is a equally active metabolite of NKH477 where one of the N-methyl groups of NKH477 is demethylated.

Cytotoxic T Lymphocyte (CTL) Assay (^{51}Cr Release Assay)

One-way MLR and CTL assays were performed as described previously (24, 25). Spleen cells from C57BL/6 mice (recipient strain) and DBA/2 mice (donor strain) were used as responder and stimulator cells, respectively. Responder cells at 5.6×10^6 were cocultured with 4.0×10^6 stimulator cells treated with mitomycin C (MMC) (final concentration 25 μg/ml) at 37°C for 20 min under 5% CO_2. Cells were cultured in 24-well plates in 2 ml of sensitization medium, namely complete RPMI 1640-10 (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 5×10^{-5} M 2-mercaptoethanol, 10% heat-inactivated FCS) containing 1 mM sodium pyruvate. NKH477 (1.5×10^{-8} M, 4.5×10^{-8} M) dissolved in saline or control vehicle only (saline) was added to the experimental cultures at the initiation of culture. After one-way MLR for 5 days, CTL activity was assessed by a ^{51}Cr release assay. DBA/2 splenic target cells (donor

TABLE 1
Median Graft Survival Time

Treatment (n)	Graft survival time of all grafts (days)	Median graft survival time (days)
Control (10)	9, 10, 10, 10, 10, 10, 10, 11, 12, 12	10
CsA 5 mg/kg/day (6)*	10, 12, 13, 13, 14, 15	13
CsA 10 mg/kg/day (6)**·†	12, 12, 13, 14, 14, 18	13.5
CsA 40 mg/kg/day (6)**·†	12, 12, 15, 16, 18, >50	15.5
NKH477 0.3 mg/kg/day (10)	9, 10, 10, 10, 11, 12, 12, 12, 14, 14	11.5
NKH477 1 mg/kg/day (10)**·†	11, 11, 11, 12, 12, 12, 14, 14, 17, 18	12
NKH477 3 mg/kg/day (10)**·†	11, 12, 13, 14, 15, 15, 16, 17, 18, 23	15

Note. Grafts survival were determined by daily abdominal palpation and regular elecrocardiogram and confirmed histologically. Rejection was defined as the day of cessation of heartbeat. Cyclosporin A (CsA) and NKH477 were orally administered at a respective dose daily.

* $P < 0.05$ vs control, **$P < 0.01$ vs control, †$P < 0.05$ vs NKH477 0.3 mg/kg/day group. No other statistically significant differences were observed between groups.

strain) or C3H/He targets (third party) were prestimulated with 10 μg/ml of LPS for 2 days and labeled with ^{51}Cr. ^{51}Cr-labeled target cells (1.0 × 10^4/well) were incubated with the C57BL/6 effector cells in quadruplicate in round-bottomed 96-well tissue culture plates at an effector-to-target ratio of 100:1, 33:1, or 11:1. After incubation at 37°C for 3.5 hr in 5% CO_2 humidified air, 100 μl of supernatant was harvested from each well and radioactivity was measured using an automatic gamma scintillation counter. Spontaneous release was less than 30% of total incorporated counts. Percentage specific cytotoxicity was calculated as

$$\% \text{ specific cytotoxicity} = \frac{(\text{cpm experiment} - \text{cpm spontaneous release})}{(\text{cpm maximal} - \text{cpm spontaneous release})} \times 100 \, (\%),$$

where spontaneous and maximum cpm were obtained by culturing target cells in medium alone and in 0.5% Triton X, respectively.

Production of IL-2 in MLR and in Mitogen Response

One-way MLR was performed in 24-well plates as described above. In the first experiment, NKH477 (4.5 × 10^{-8} M) dissolved in saline or saline only (control) was added in a volume of 20 μl to the experimental cultures at the initiation of culture. The supernatants were harvested serially at 12, 24, 48, 72, or 120 hr after initiation and stored at −70°C until enzyme-linked immunosorbent assay (ELISA). In the second, NKH477 dissolved in saline or stock solution of CsA in dimethyl sulfoxide was diluted with RPMI 1640 and added in a
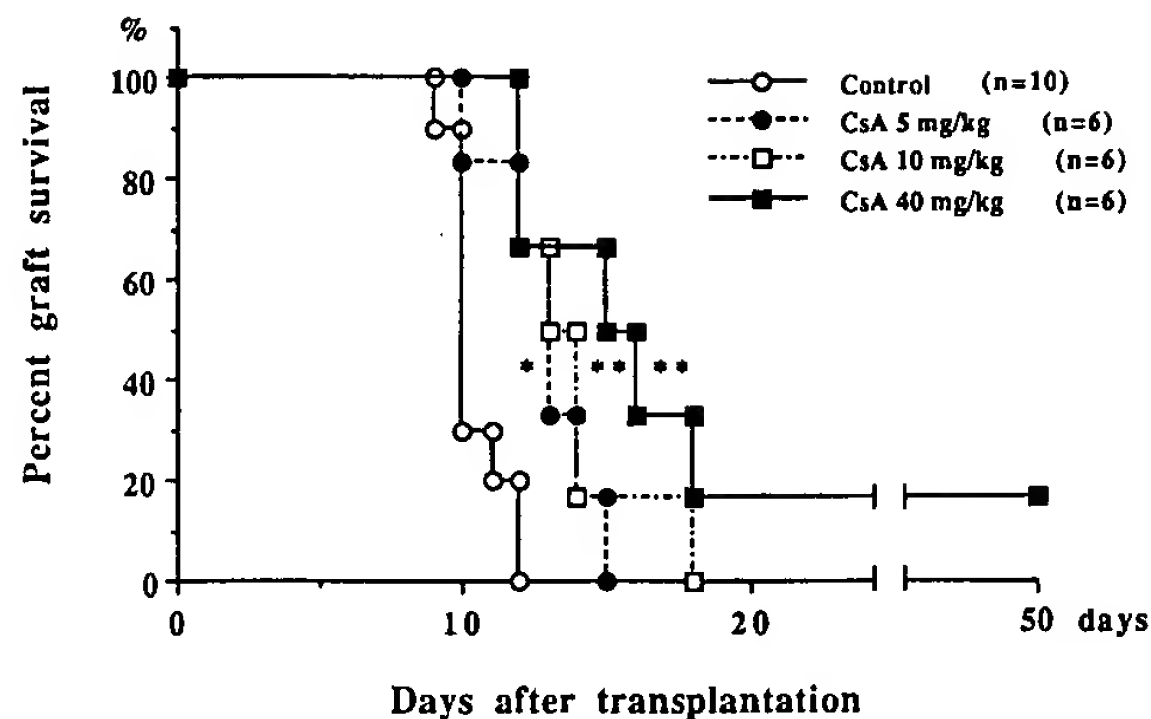
FIG. 1. Effects of cyclosporin A (CsA) on cardiac allograft survival. Oral administration of CsA at 5, 10, or 40 mg/kg/day provided slight to mild prolongation of cardiac allograft survival in comparison with control group, whereas no significant differences of efficacies were observed among treated groups. *$P < 0.05$ vs control, **$P < 0.01$ vs control.

volume of 40 μl to the cultures at the initiation of MLR. The final concentration of NKH477 was 1.5 × 10^{-8}, 4.5 × 10^{-8}, or 13.5 × 10^{-8} M and CsA was 1 × 10^{-10}, 1 × 10^{-9}, 1 × 10^{-8}, or 1 × 10^{-7} M. After 48 hr MLR, the supernatants of the cultures were harvested and stored at −70°C until ELISA. In the third experiment, spleen cells (5 × 10^6 cells/ml) from C57BL/6 mice were incubated with 4 μg/ml of Con A in a volume of 1 ml/well for 24 hr in the presence of NKH477 or CsA at the concentrations same as the second experiment in 24-well plates. The supernatants of the cultures were harvested and stored at −70°C until ELISA. As controls, same volumes of RPMI were added to the cultures as substitutes for NKH477 and CsA. Murine IL-2 in the supernatants was quantified using ELISA systems purchased from Endogen (Boston, MA). The sensitivity of the system was >0.03 units/ml. All measurements were performed in duplicate.
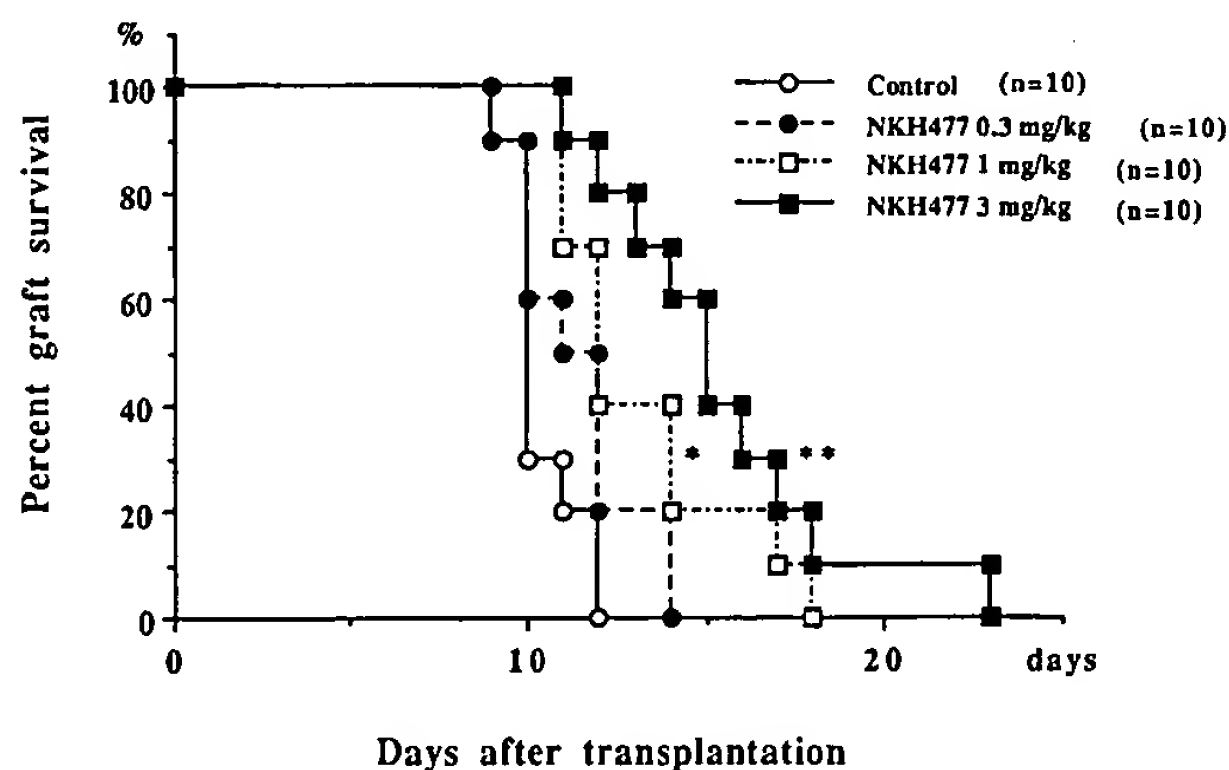
FIG. 2. Effects of NKH477 on cardiac allograft survival. Oral administration of NKH477 at 1 or 3 mg/kg/day provided slight or mild prolongation of cardiac allograft survival, whereas NKH477 at 0.3 mg/kg/day failed to prolong graft survival. *$P < 0.01$ vs control, **$P < 0.01$ vs NKH477 0.3 mg/kg/day group.
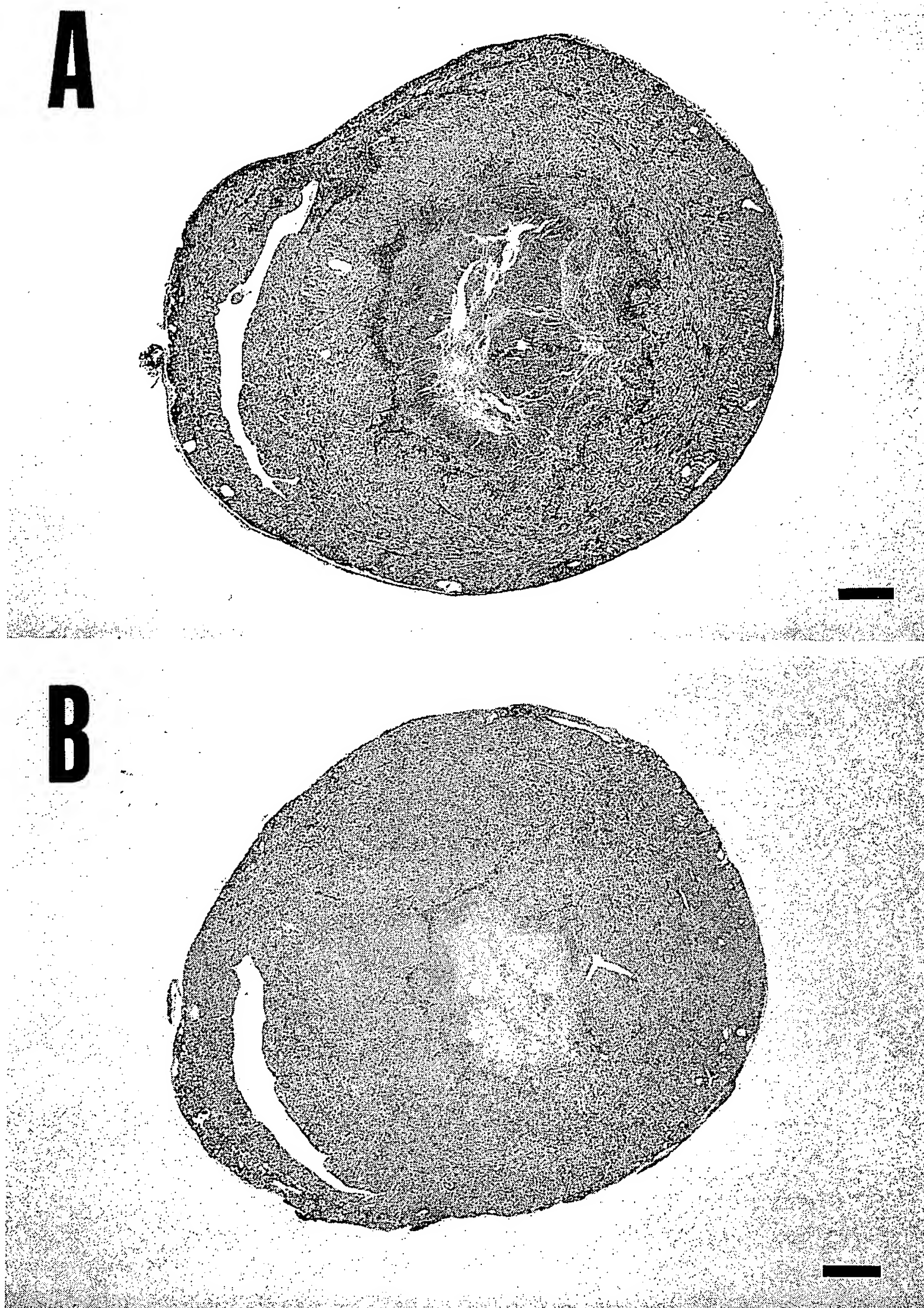
FIG. 3. Histopathology of mouse cardiac allografts. (A) Low-power photomicrograph from a vehicle-treated DBA/2 → C57BL/6 allograft. The graft was examined on Posttransplant Day 5. Note prominent interstitial mononuclear cell infiltration. (B) A DBA/2 → C57B/6 allograft treated with NKH477 at 3 mg/kg/day and examined on Posttransplant Day 5. Inflammatory changes in the graft were less severe. Bar: 500 μm. (C) High-power photomicrograph of a vehicle-treated allograft [same sample as (A), LV posterior wall]. (D) High-power photomicrograph of an NKH477-treated allograft [same sample as (B), LV posterior wall] (hematoxylin and eosin; bar: 50 μm).
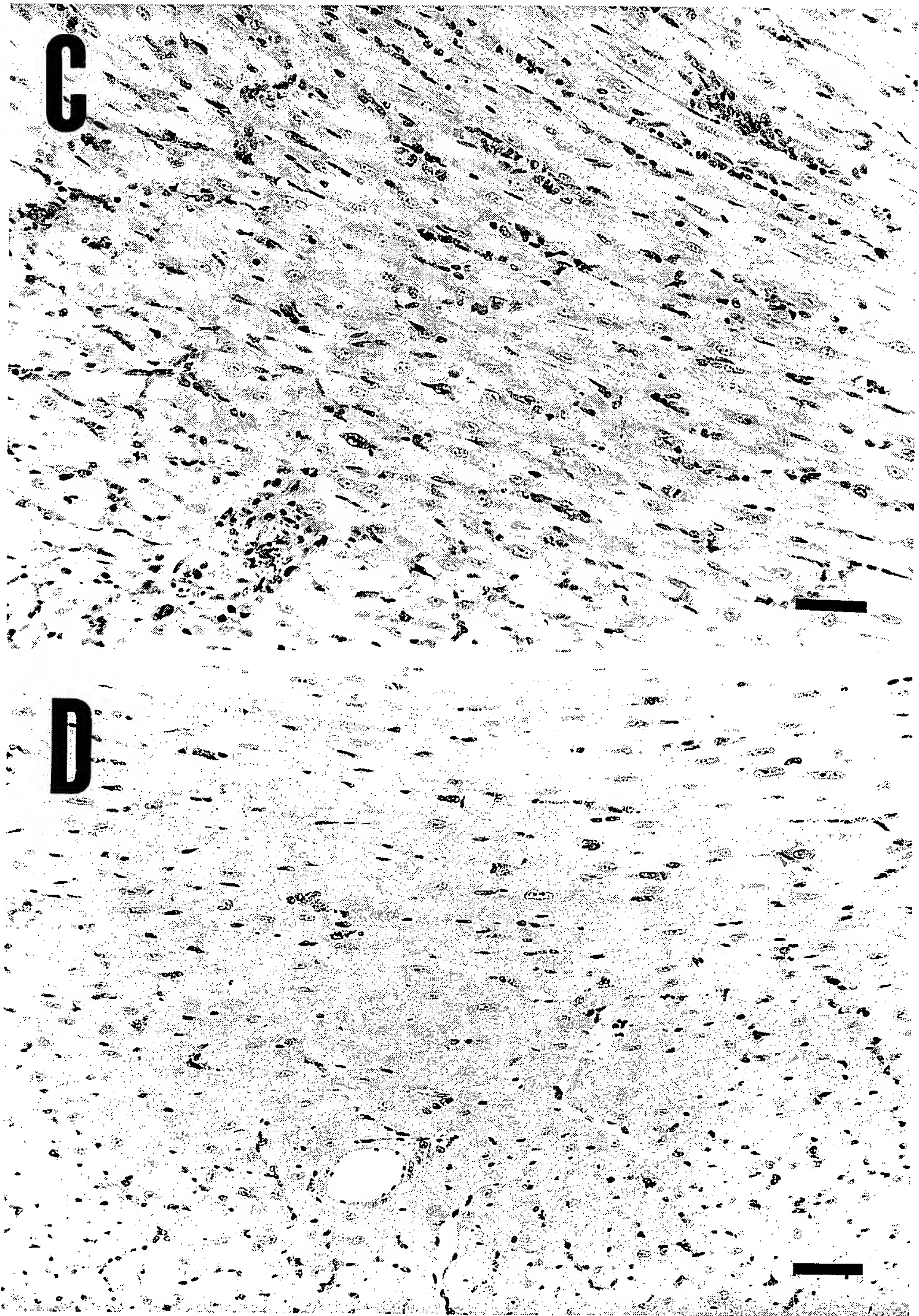
FIG. 3—*Continued*

TABLE 2
Histopathological Scores of Cardiac Allografts[a]

Treatment	n	Myocardial inflammation	Perivascular inflammation	Necrosis
Control	6	1.92 ± 0.20	1.75 ± 0.11	1.25 ± 0.11
NKH477[b]	5	1.20 ± 0.12*	1.30 ± 0.12*	1.30 ± 0.20

[a] Lesions graded as described under Methods by two blinded observers. Data are expressed as means ± SEM, and compared by Mann–Whitney U test.

[b] NKH477 was administered orally at a single daily dose of 3 mg/kg for 5 days.

* $P < 0.05$ vs control.

[³H]Thymidine Incorporation Assay

Spleen cells from C57BL/6 mice (recipient strain) were suspended at 1.0×10^8/ml in culture medium. They were passed through nylon wool columns purchased from Wako Chemical Co. (Osaka, Japan) to enrich splenic T cells by a modification of the method established by Julius et al. (26) following the manufacturer's instructions. The obtained cells were subjected to cytotoxic elimination test using anti-Thy-1.2 mAb and low toxic rabbit serum complement, and shown to include 73 to 80% T cells. These enriched splenic T cells were treated in one of three ways. In the first treatment, cells were resuspended in sensitization medium and incubated at 2×10^5 cells/well with 4×10^5 cells/well MMC-treated spleen cells from DBA/2 mice in 6 wells for each treatment. NKH477 (4.5×10^{-8} M) diluted in RPMI 1640 or control vehicle (RPMI 1640) was added in a volume of 10 μl at the initiation of culture. On Day 5, 1 μCi of [³H] thymidine was added to each well, and the cells were harvested 6 hr later. In the second treatment, cells were resuspended in complete RPMI 1640-10 and incubated at 2×10^5 cells/well in 4 wells. Recombinant human IL-2 (rhIL-2) at 1 nM (45
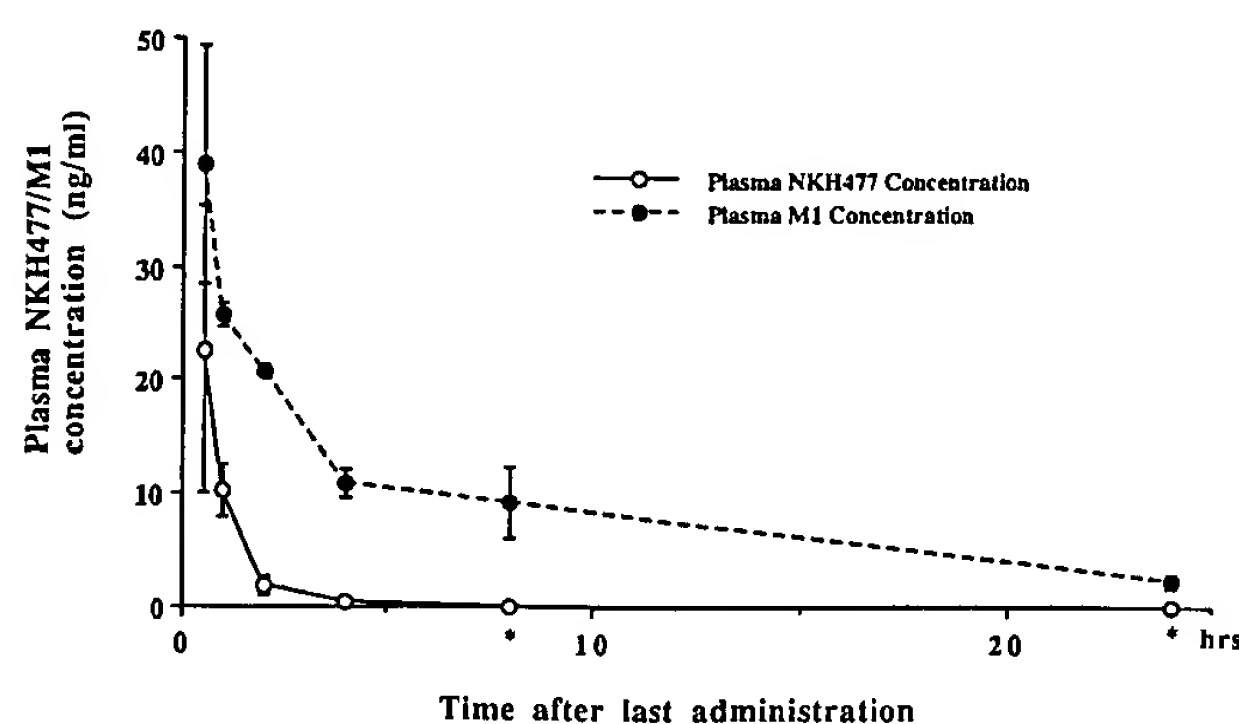
FIG. 4. Pharmacokinetics of NKH477 and its active metabolite M1 in mice. Mice were orally administered NKH477 at 3 mg/kg for 5 consecutive days. Thirty minutes, 1, 2, 4, 8, and 24 hours after last administration, three mice at each point were killed and plasma was collected. Plasma concentration of compounds were measured by gas chromatography/mass spectrometry. Values are expressed as means ± SEM. *Not detectable.
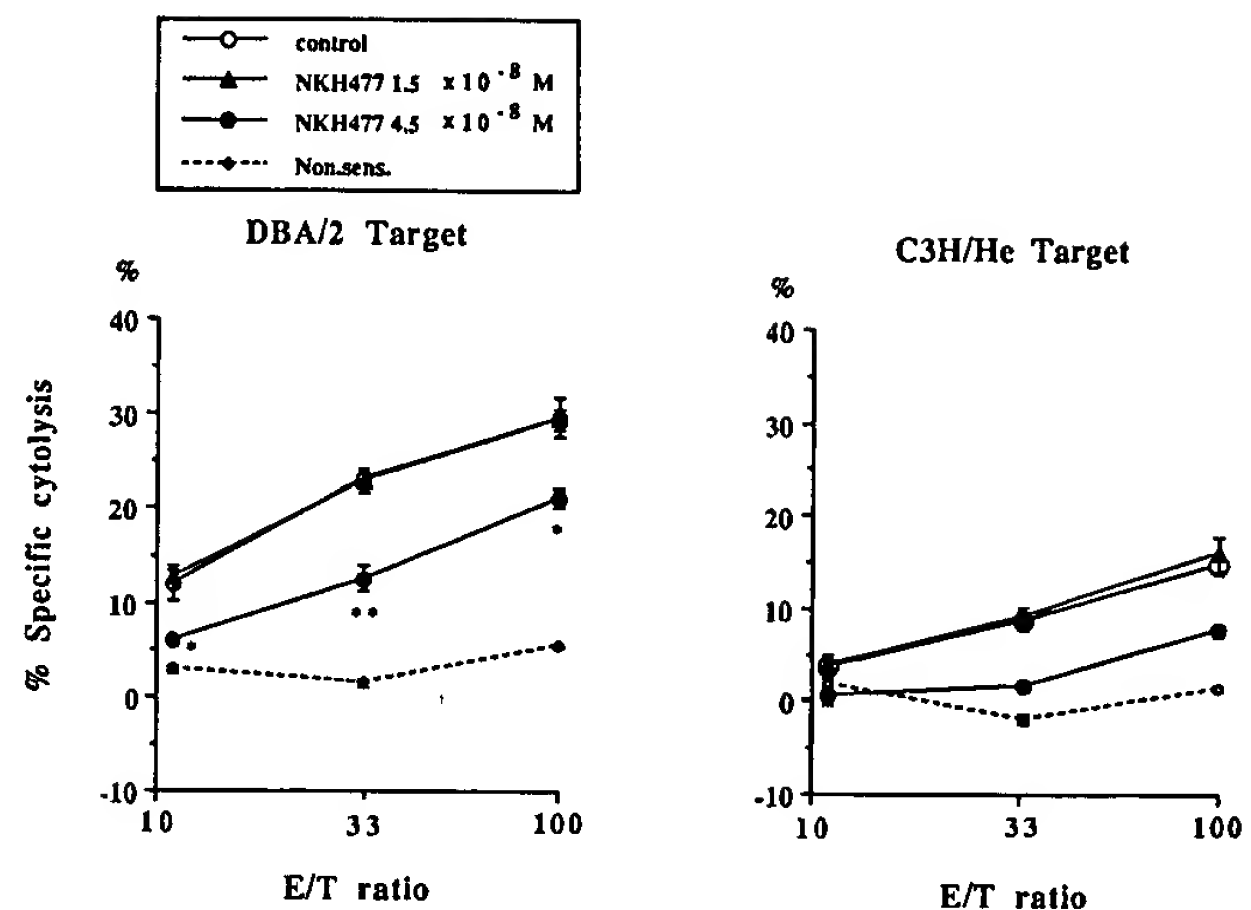
FIG. 5. Inhibition of CTL generation in vitro by treatment with NKH477. Splenocytes from C57BL/6 mice were stimulated with DBA/2 cells for 5 days, and then tested against DBA/2 or C3H/He cells. The graphs represent cytolytic activities at the effector to target ratios of 11, 33, and 100. NKH477 was added to the medium during the 5-day coculture at a concentration of 1.5×10^{-8} or 4.5×10^{-8} M. Control C57BL/6 splenocytes were sham-treated with the vehicle only. Non. sens. means nonsensitized control. Values are expressed as means ± SEM of quadruplicate cultures. *$P < 0.05$, **$P < 0.01$ vs control.

units/ml) or control vehicle (RPMI 1640), and NKH477 (4.5×10^{-8} M) diluted in RPMI 1640 or control vehicle (RPMI 1640) was added at the initiation of culture. After incubation for 42 hr, [³H]thymidine (1 μCi) was added to each well and, after a 6-hr pulse, the cells were harvested. In the third, cells were resuspended in sensitization medium and one-way MLR was performed in 24-well culture plates as described above. On Day 4 of culture, primed cells were harvested and centrifuged. After three washes with culture medium, the viable cells were resuspended in cRPMI 1640-10 and incubated at 2×10^5/well in 5 wells with or without 1 nM of rhIL-2 for 18 hr. Then, after a 6-hr pulse with [³H]thymidine (1 μCi/well), the cells were harvested. For all harvested cells, [³H]thymidine incorporation was assessed by scintillation counting. All cultures except those for priming MLR were performed in a volume of 200 μl in round-bottomed 96-well plates.

Statistical Analysis

The significance of differences between two nonparametric groups in the allograft survival experiments and scores for histopathological findings were examined by the Mann–Whitney U test. Percentage cytolysis in CTL assay, levels of IL-2 in culture supernatants, and [³H]thymidine incorporation were compared by one-factor ANOVA followed by Fisher's protected least significant difference.
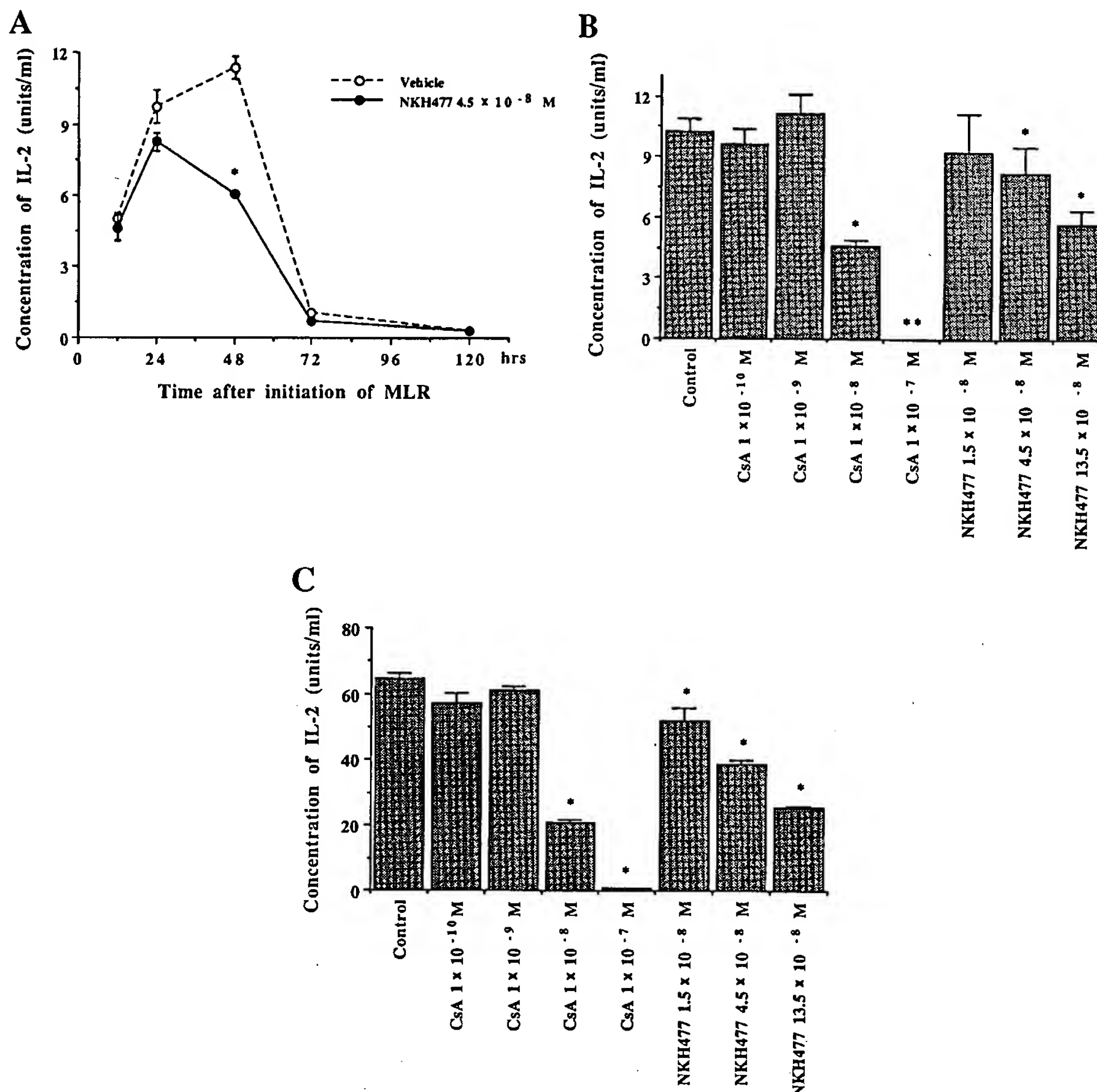
FIG. 6. (A) Serial IL-2 production during allogeneic MLR incubated in the presence of NKH477 or control vehicle. NKH477 was added to the medium at a concentration of 4.5×10^{-8} M from the initiation of MLR. Supernatants were collected serially and the concentrations of IL-2 were assayed by ELISA. Values are expressed as means ± SEM of 4 wells. (B) Bar graphs indicate concentrations of IL-2 in the supernatants after 48 hr MLR in the presence of several doses of NKH477 or CsA. NKH477 suppressed IL-2 production during MLR in a dose-dependent manner, although it was less potent than CsA at the concentrations equivalent to the plasma level in clinical use. Values are expressed as means ± SD of 4 wells. (C) Concentrations of IL-2 in the supernatants were quantified after 24 hr mitogen stimulation by 4 μg/ml of Con A in the presence of several doses of NKH477 or CsA. NKH477 and CsA suppressed IL-2 production with dose dependencies similar to the case of MLR. Values are expressed as means ± SD of 4 wells. *$P < 0.01$ vs control. **Values were lower than the minimal sensitivity (0.03 units/ml).

RESULTS

Effect of NKH477 and Cyclosporin A on Cardiac Allograft Survival

During the experiments, recipient mice appeared healthy and no differences in body weight were observed among the NKH477-treated, CsA-treated, and saline-treated control groups. In this acute rejection model, saline-treated C57BL/6 recipient mice rejected all DBA/2 cardiac allografts within 12 days with a median survival time of 10 days. Oral administration of a low dose (5 or 10 mg/kg/day) of CsA prolongs graft survival only slightly and 40 mg/kg/day of CsA exhibited only mild but significant effect on graft survival with a median survival time of 15.5 days (Table 1, Fig. 1). In NKH477-treated groups, graft survival was slightly prolonged in mice treated with NKH477 at 1 mg/kg/day to a median survival time of 12 days and mildly prolonged in those treated at 3 mg/kg/day to a median
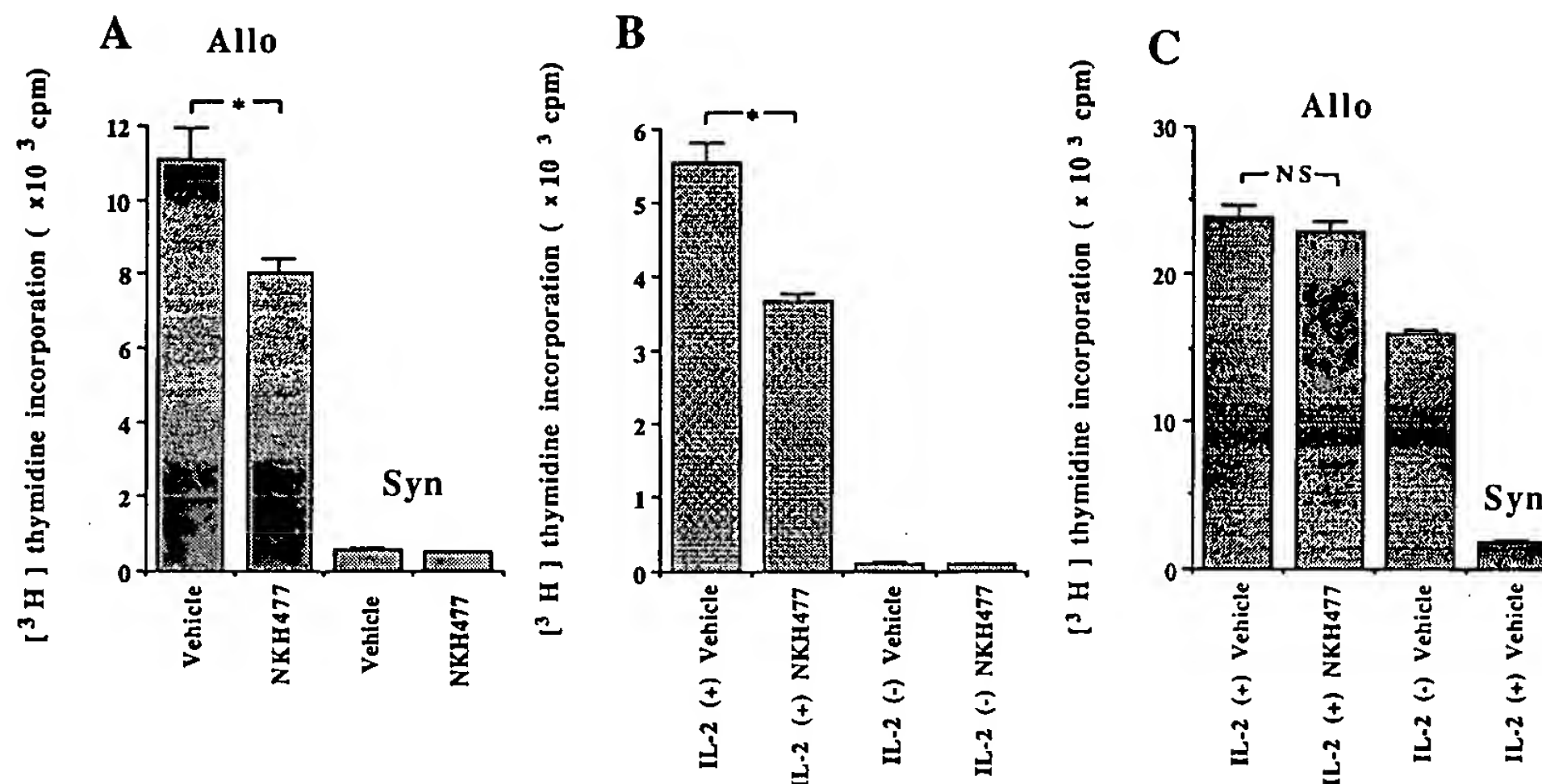
FIG. 7. Effects of NKH477 on [³H]thymidine incorporation by T cell-enriched spleen cells. (A) Splenic T cells from C57BL/6 mice (recipient strain) were cocultured with MMC-treated spleen cells from DBA/2 mice (donor strain, allo) or C57BL/6 (syn). On Day 5 of MLR cells were pulsed for 6 hr with [³H]thymidine (1 μCi/well). (B) Unprimed splenic T cells from the recipient strain were stimulated to proliferate with exogenous IL-2 (1 nM). Cells were also cultured without addition of IL-2 to exclude the effect of NKH477 itself on proliferation. (C) Splenic T cells were primed by one-way MLR (allo; allogeneic, syn; syngeneic) and after washing, cells were stimulated with exogenous IL-2. Values are expressed as means ± SEM. *$P < 0.01$.

survival time of 15 days ($P < 0.01$ vs saline-treated control; Mann–Whitney U test). However, a low dose of NKH477 (0.3 mg/kg/day) failed to significantly prolong graft survival (median graft survival time 11 days; Table 1, Fig. 2).

Histopathological Examination

As CTL assay confirmed the existence of CTL after MLR for 5 days, histopathological examination was performed in DBA/2-C57BL/6 cardiac allografts harvested 5 days after transplantation. The grafts of the NKH477-treated group (3 mg/kg/day) were compared with those of the saline-treated control group. As shown in Fig. 3, inflammatory changes in the grafts appeared less prominent in the NKH477-treated group. Moreover, the scores for myocardial and perivascular inflammation were significantly lower in the NKH477-treated group ($P < 0.05$). There were no statistically significant differences between the scores for necrosis of the two groups (Table 2).

Pharmacokinetics of NKH477 and Its Active Metabolite M1

Plasma concentrations of NKH477 and M1 were 22.68 ± 12.65 and 39.01 ± 10.46 ng/ml (mean ± SEM), respectively, 30 min after last administration of 3 mg/kg of NKH477. They then declined with half-lives of 0.43 and 1.81 hr, respectively. Twenty-four hours after last administration, no NKH477 and little M1 were detected in the plasma of mice (Fig. 4).

Effect of NKH477 on the Generation of Antigen-Specific CTL

To examine *in vitro* one of the mechanisms of the above findings in allograft survival, we investigated the effect of NKH477 on CTL activity. Specific cytolysis by C57BL/6 splenocytes (recipient strain) of DBA/2 splenocytes (donor strain) and of C3H/He splenocytes (third strain) was measured (Fig. 5). C57BL/6 cells treated with control vehicle and cells treated with 1.5 × 10⁻⁸ M NKH477 showed similar increases in CTL response to DBA/2 target cells after MLR for 5 days. In contrast, cells treated with 4.5 × 10⁻⁸ M NKH477 during MLR showed decreased CTL activity. These C57BL/6 cells showed little cytolytic activity when the target cells were from a third strain. Further, nonsensitized C57BL/6 spleen cells also showed little cytolytic activity against DBA/2 target cells.

Effect of NKH477 and CsA on IL-2 Production

In the first experiments, supernatants of allogeneic MLR were harvested serially at 12, 24, 48, 72, or 120 hr after initiation, and concentrations of IL-2 were quantified. As shown in Fig. 6A, addition of NKH477 at a concentration of 4.5 × 10⁻⁸ M to the culture significantly suppressed IL-2 production at 48 hr after initiation (NKH477 treated vs control vehicle: 6.05 ± 0.13 vs 11.35 ± 0.46; mean ± SEM units/ml; 46.6% suppression) when the control culture exhibited peak production. Figure 6B demonstrates the effects of several concentrations of NKH477 or CsA on the IL-2 production after 48 hr MLR. CsA showed almost complete inhibi-

tion at the equivalent concentration (1×10^{-7} M) to the therapeutic plasma level. Addition of NKH477 in the culture medium suppressed IL-2 production dose dependently, although the efficacy of NKH477 was relatively weak in comparison with CsA. NKH477 and CsA also exhibited similar inhibition patterns to the MLR, respectively, on IL-2 production during mitogen response to Con A (Fig. 6C).

Effect of NKH477 on T Cell Proliferation

[^{3}H]Thymidine incorporation was significantly inhibited by NKH477 at a concentration of 4.5×10^{-8} M (80,056 ± 3656, mean cpm ± SEM) compared with the control vehicle (111,175 ± 8282) in allogeneic MLR with a percentage inhibition of 28.0% and in IL-2 stimulation of unprimed splenic T cells (NKH477 vs control vehicle: 36,463 ± 1151 vs 55,565 ± 2694) with a percentage inhibition of 34.4% (Figs. 7A and 7B). However, the drug at the same concentration could not significantly suppress the proliferative response to exogenous IL-2 of cells primed and activated by allogeneic MLR (NKH477 vs control vehicle: 229,344 ± 6381 vs 238,636 ± 7966) (Fig. 7C).

DISCUSSION

We investigated the effects of NKH477 on cardiac allograft survival and on histopathological findings in the early stage of acute cellular rejection. In this acute rejection model, all DBA/2 cardiac allografts in saline-treated C57BL/6 recipients without any immunosuppression were rejected within 12 days. Lower doses of CsA (5 or 10 mg/kg/day) had only slight effect on graft survival and 40 mg/kg/day of CsA had mild effect in this model. Considering body surface area, the oral dose of 40 mg/kg/day in mice is comparable to 3 mg/kg/day in human (27) and this dose is the maintenance dose of CsA in clinical cardiac transplantation. Treatment with NKH477 at 1 mg/kg/day beginning on the day of transplantation resulted in slight prolongation of allograft survival, and at 3 mg/kg/day in a mild prolongation of survival with a similar median survival time to that of the maintenance dose of CsA (Table 1). Inflammatory changes were less prominent in animals treated with NKH477 at 3 mg/kg/day than in the vehicle-treated group. Although no significant difference between the two groups was seen in the score for necrosis, it seems to be the reason that necrosis becomes apparent later in the course of rejection.

cAMP-elevating agents such as forskolin have been suggested to have immunosuppressive effects on lymphocytes. For example, they have an inhibitory effect on IL-2-secreting Th1 lymphocytes in vitro (1), and decrease IL-2 production and IL-2Rα (IL-2 receptor α-chain) expression via the suppression of both protein product and mRNA levels (2). Blockade of the IL-2 pathway suppresses immunological responses to al-

loantigens and prevents acute allograft rejection (28–30). The increase in [cAMP]$_i$ induced by forskolin and other agents is related to the inhibition of T cell proliferation (1, 31, 32). It also leads to the inhibition of CTL activity (4, 5). While CTL, activated and induced to proliferate after stimulation by alloantigens, are considered to play an important role in the course of allograft rejection (7–10), CTL generated in the presence of forskolin showed suppressed lytic activity but normal proliferative responses to alloantigens (5).

Considering these reports and the results of in vivo experiments, we tried to clarify the effects of NKH477 on alloreactive CTL generation and IL-2 production during MLR and mitogen response to Con A. We compared its inhibitory effect on IL-2 production to that of a standard immunosuppressant CsA, since CsA is considered to exhibit immunosuppressive effects mostly via inhibition of IL-2 gene transcription.(33–35) We used a concentration of NKH477 equivalent to the plasma level in in vivo experiment (Fig. 4). NKH477 at 4.5×10^{-8} M (24.6 ng/ml) inhibited the generation of alloreactive CTL and suppressed IL-2 production during MLR and mitogen response; however, it was less effective than equivalent concentration of CsA to the therapeutic plasma level. Prolongation of cardiac allograft survival might have resulted from this suppression of IL-2 production, reflecting elevated expression of cytokines such as IL-2 in organ allografts undergoing acute rejection (36). These results also suggest some possible mechanisms by which NKH477 may have inhibited the generation of CTL. NKH477 might first suppress IL-2 production, and the resulting decrease in IL-2 concentration may inhibit the differentiation of CTL. NKH477 might inhibit T cell proliferation through the decreased production of IL-2 or through the reduced responsiveness to IL-2 of T cells (including CTL precursor), thereby leading to inhibition of CTL generation, although other humoral factors for CTL generation have not been investigated in this study. We therefore assessed whether NKH477 had inhibitory effects on T cell proliferation during MLR and on the proliferative responses to IL-2 of primed and unprimed splenic T cells using [^{3}H]thymidine incorporation. The results indicated that NKH477 had an inhibitory effect on T cell proliferative response when present in the culture medium throughout MLR or when unprimed T cells were stimulated with IL-2 in the presence of this drug. However, it did not have an inhibitory effect on IL-2 responsiveness of alloantigen-primed T cells at its physiological dose. These results indicate that NKH477, a cAMP-elevating agent, cannot block the progression of alloantigen-primed T cell proliferation at its physiological dose, although the possibility remains that it may counteract the initiation of T cell proliferation.

The combined concentration of NKH477 and its equally active metabolite M1 in the plasma were kept

higher than 4.5×10^{-8} *M*, the concentration in the culture medium in *in vitro* experiments, at 4 hr after the last administration. This suggests that immunosuppressive effects of NKH477 in the *in vivo* experiments were exerted through modulation of T cell functions such as inhibition of CTL generation, while the drug could not have a suppressive effect on the proliferative response of primed T cells and the effects might not be specific to the suppression of allo-reactive immunity.

Valitutti *et al.* reported that increases in $[cAMP]_i$ suppressed the adhesion and motility of CTL, possibly by regulation of elements of their cytoskeleton formation such as the F-actin and tubulin network (6). Thus adenylate cyclase activators inhibit functions of generated CTL. Improved histopathological findings in the early stages of NKH477-treated allografts are also suggestive of these effects. In addition, recent studies have shown that cyclic AMP analogs and cAMP-elevating agents enhance cardiac preservation in rat and baboon cardiac transplant models through their beneficial effect on vascular homeostatic mechanisms during global ischemia (37, 38). NKH477 may improve oxygen supply to ischemic regions of allografts undergoing rejection by maintaining vascular functions(possibly endothelial function) (37), thereby prolonging graft survival. Another beneficial effect of NKH477 on vascular function is its vasodilating effect in coronary arteries. In smooth muscle cells of coronary arteries, NKH477 attenuates acetylcholine-induced Ca^{2+} mobilization and reduces the sensitivity of contractile machinery to Ca^{2+}, possibly by activating cAMP-dependent mechanisms (39).

In conclusion, NKH477, a water-soluble forskolin derivative, has inhibitory effects on antigen-specific CTL activities, and other beneficial effects on cardiac allograft survival. Concerning inhibitory effects on IL-2 production, NKH477 was less effective than CsA and the drug might have less efficacy on *in vivo* immunosuppression than moderate to high doses of CsA. However, considering that NKH477 also has a positive inotropic effect on failing hearts, this drug may be a promising agent for the management of hemodynamic states of patients after cardiac transplantation and for therapy against the cardiac dysfunction which emerges as a manifestation of acute rejection. Further studies are required to assess the beneficial effects of this drug and roles of immunomodulation by inotropic agents.

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RAPID COMMUNICATION

Combination Therapy with a CD4-CDR3 Peptide Analog and Cyclosporin A to Prevent Graft-vs-Host Disease in a MHC-Haploidentical Bone Marrow Transplantation Model[1]

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Graft-versus-host disease (GVHD) is a major complication associated with allogeneic bone marrow transplantation (BMT). Cyclosporin A (CsA) has been used as the basis for most immunosuppressive regimens for the prevention of GVHD, but has exhibited only limited effects and is hampered by nephrotoxicity, neurotoxicity, and hepatotoxicity. Previously, we showed that rD-mPGPtide, a structure-base designed peptide analog of the CDR3-like region of domain 1 of the murine CD4 molecule, suppressed the development of GVHD in a MHC-haploidentical murine BMT model when administered early in the course of disease. This peptide analog also inhibited T cell proliferation in mixed lymphocyte reactions (MLR) *in vitro*. The current results demonstrate that CsA and rD-mPGPtide exhibit an additive inhibitory effect on MLR. Furthermore, the use of CsA and rD-mPGPtide together for prevention of GVHD nearly doubled the median survival time of the mice compared to either agent alone. In addition, the combination therapy reduced the requirement for habitual administration of CsA. Therefore, the use of a CD4-CDR3 peptide can complement and potentiate the immunosuppressive effects of CsA in the prevention of GVHD following allogeneic BMT. © 1998 Academic Press

Key Words: **rD-mPGPtide; CD4 peptide; cyclosporin A; graft-vs-host disease; bone marrow transplantation.**

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is currently being used as a treatment for a number of disease states including leukemias, multiple myeloma myelodysplasia, aplastic anemia, and severe combined immunodeficiency (1). The major complications which impede the overall success of this treatment include the development of graft-versus-host disease (GVHD),

failure to engraft, infections resulting from chronic immunoincompetence, and leukemic relapse. Acute GVHD is caused by contaminating mature donor T cells in the bone marrow graft and leads to significant morbidity and mortality. The inhibition of T cell responses following BMT, by various means, has proven to be effective in preventing GVHD. Cyclosporin A (CsA), which has moderate effects on GVHD, has become the basis for many of the immunosuppressive regimens (1).

CsA has been shown to be a potent immunosuppressive agent for solid organ transplantation and is currently being tested for treatment of several autoimmune diseases (2). CsA is thought to specifically inhibit lymphocytes by binding to cyclophilin (CyP), and this CsA–CyP complex binds to and blocks the function of calcineurin. Calcineurin, a phosphatase, plays a critical role in the signaling cascade of T cells leading to the production of cytokines such as interleukin-2 (IL-2) (5, 6). Although CsA has been successfully used for the treatment of GVHD following allogeneic BMT, it has associated drawbacks, including hepatotoxicity, neurotoxicity, and nephrotoxicity (1, 2). Previous studies have also indicated that CsA can interfere with the normal thymic development of T cells leading to immunoincompetency and the potential generation of autoreactive T cells (3). Equally important is the need to administer frequent doses of CsA to patients for up to 6 months or more post-BMT, since alloreactive donor T cells can survive treatment and become activated once immunosuppression is halted (1).

Our earlier reports documented that the CD4-CDR3 peptide analog, rD-mPGPtide, was a potent inhibitor of murine CD4 T-cell-mediated immune responses, both *in vitro* and *in vivo* (4, 7, 8). In this regard, we also demonstrated that rD-mPGPtide suppressed the *in vivo* alloreactive responses associated with the onset of GVHD in the (B6 × DBA2)F$_1$ → (B6 × CBA)F$_1$ MHC-haploidentical irradiated BMT model (8). In this model, the donor and recipient mice possess a full MHC mismatch, involving both class I and class II differences.

Injection of irradiated (950 cGy) (B6 × CBA)F₁ mice with an inoculum of donor T cells along with donor bone marrow induced an acute GVHD reaction which resulted in fatality within 2-3 weeks. The current study indicated that rD-mPGPtide and CsA could inhibit T cell responses *in vitro* and were more potent together than either agent was alone. Furthermore, administration of rD-mPGPtide to recipient mice following BMT significantly increased the median survival time (MST) of mice with GVHD, as did the administration of CsA. However, when rD-mPGPtide and CsA were administered together, survival was greater than when either agent was administered alone.

MATERIALS AND METHODS

Mice. Mice, (B6 × DBA2)F₁ [(B6D2)F₁] (H-2^{bd}) and (B6 × CBA2)F₁ [(B6CBA)F₁] (H-2^{bk}), were purchased from The Jackson Laboratory (Bar Harbor, ME). Male mice were used as donors between the ages of 7 and 12 weeks and as recipients between the ages of 9 and 16 weeks. Mice were kept in a sterile environment in microisolators at all times and were provided with acidified water and autoclaved food.

Media. Buffered saline solution (BSS) supplemented with 0.1% BSA (Hyclone, Logan, UT) was used for all *in vitro* manipulations of the donor bone marrow and lymphocytes. For injection, cells were resuspended in BSS alone. RPMI 1640 (Mediatek, Herndon, VA) supplemented with 10% FCS (Sigma, St. Louis, MO) and 10 U/ml glutamine, 10 U/ml penicillin and streptomycin, and 0.05mM BME (Mediatech, Inc. Herndon, VA) was used for all *in vitro* mixed lymphocyte responses.

Peptides. The peptides were designed as previously described (7), synthesized on an Applied Biosystems, Inc. (Foster City, CA) 430A peptide synthesizer using standard Fmoc chemistry, refolded to enrich for intramolecular disulfide bonding, and purified by HPLC (Waters 600E system controller, Waters 490E programmable multiwavelength detector, Millipore Corp, Bedford, MA) before use. The sequences of the synthesized peptides were as follows: rD-mPGPtide (CPG-PEEKRNELEC, all D-amino acids) and scrambled rD-mPGPtide (Scr-PGPtide; same amino acid composition, but scrambled sequence, CEPKNELPERGEC, all D-amino acids). For treatment of GVHD, peptides were reconstituted in BBS and injected at the appropriate dose and time into mice iv in a volume of 0.25 ml.

Injections. All cell suspensions were given intravenously via the tail vein in a maximum volume of 0.5ml of BSS.

Irradiation. All recipient mice received a 950-cGy exposure from a Gammacell ¹³⁷Cs source (116 cGy/min).

mAbs. Ascites fluid for anti-Thy-1.2 (J1j, rat IgM) and anti-CD8 (3.168, rat IgM) mAb were used for cell preparations. In addition, goat anti-mouse IgG (whole molecule) antibodies were purchased from Cappel-Organon Teknika (Westchester, PA). Guinea pig serum prepared in our laboratory was used as a source of complement C for all mAb treatments.

Preparation of cells. Bone marrow cells were obtained from the femurae and tibiae of donor mice by flushing with BSS w/0.1% BSA. To prepare anti-Thy-1-treated (T cell depleted) bone marrow (ATBM), cells were incubated with J1j mAb (at 1:100 dilution) and C (1:25) for 45 min at 37°C and were washed four times. T-cell-enriched donor cell populations were prepared by treating pooled spleen and lymph node cells with: (a) Gey's balanced salt lysing solution containing 0.7% NH₄Cl for removal of RBC; and (b) panning on a plastic petri dish precoated with a 5 μg/ml solution of goat anti-mouse IgG for 1 h at 37°C to remove B cells. These treatments resulted in populations of 90–95% CD3⁺ cells, as quantitated by flow cytometric analysis. Further purification of T cells into CD4⁺ cells was performed as described previously. These procedures resulted in purified populations of CD4⁺ cells (>90%) with no detectable presence of the inappropriate subset.

Mortality assay for GVHD. Recipient mice were irradiated with 950 cGy and approximately 6 h later were injected intravenously with either donor ATBM (2 × 10⁶) alone as a negative control or a mixture of ATBM plus donor T cells as indicated. Mice were checked daily for morbidity and mortality until the experiments were terminated at day 60 after transplantation. MST were calculated as previously described. Statistical comparisons between experimental groups for mortality curves were performed by the nonparametric Wilcoxon signed rank analysis.

RESULTS

The effect of rD-mPGPtide and cyclosporin A on alloreactivity in vitro. To assess the effect of combining rD-mPGPtide and CsA on T cell responses *in vitro*, lymph node cells taken from (B6 × DBA2)F₁ mice were stimulated *in vitro* with irradiated (15 Gy) spleen cells from (B6 × CBA)F₁ mice. The proliferative response was measured by pulsing with [³H]TdR for the final day of a 5-day culture. Titered concentrations of rD-mPGPtide (50–200 μM) were added to appropriate culture wells in order to test the inhibitory properties of the peptide. As indicated in Table 1a, rD-mPGPtide inhibited proliferation in a dose dependent manner. Nearly 90% inhibition of the proliferative response was observed with the addition of 200 μM rD-mPGPtide, and the 50% inhibitory concentration (IC₅₀) was approximately 100 μM. CsA was also a potent inhibitor

TABLE 1

In Vitro Inhibition of MLR by a Combination of rD-mPGPtide with CsA

rD-mPGPtide (μM)	CsA (μg/ml)			
	0	0.001	0.01	0.1
(A) (B6xDBA2)F$_1$ Anti-(B6xCBA)F$_1$				
0	31.5 ± 3.3	23.1 ± 1.2	7.5 ± 3.6	ND
50	24.8 ± 13.6	19.5 ± 0.8	9.0 ± 9.1	ND
100	15.6 ± 4.7	12.9 ± 5.3	3.5 ± 1.0	ND
200	4.2 ± 0.8	2.7 ± 1.2	2.7 ± 0.5	ND
(B) CBA Anti-C57BL/6				
0	14.1 ± 3.0	ND	9.7 ± 0.5	4.2 ± 1.4
17	7.7 ± 1.8	ND	6.0 ± 1.1	2.1 ± 0.7
33	5.9 ± 2.7	ND	3.6 ± 0.9	2.8 ± 0.6
67	2.5 ± 1.4	ND	1.5 ± 0.3	1.0 ± 0.7

Note. Data are expressed as a response index ± SD. Response index is defined as the ratio of the [^{3}H]TdR incorporated by experimental cells and the [^{3}H]TdR incorporated by unstimulated responder cells. ND, no data. In each well, 2 × 10^5 responder cells were stimulated with 4 × 10^5 irradiated stimulator cells. Wells were pulsed with 1 μCi [^{3}H]TdR.

of the MLR in the range of 1–10 ng/ml. In addition, the peptide and CsA exhibited an additive effect on inhibiting the proliferative response of the MLR, i.e., suppression was greater when the reagents were added together than with either one alone. This additive effect was also observed in MLR when other strain combinations were utilized (Table 1b).

The effect of combined treatment of rD-mPGPtide and cyclosporin A on GVHD. In an attempt to evaluate the effect of combining rD-mPGPtide and CsA *in vivo*, an acute form of GVHD was induced in lethally irradiated (9.5 Gy) (B6 × CBA)F$_1$ mice by administration of 5 × 10^6 (B6 × DBA2)F$_1$ T cells along with 2 × 10^6 (B6 × DBA2)F$_1$ ATBM. In this GVHD model, the untreated mice exhibited 80% fatality by day 20 posttransplant with a MST of 13 days (Fig. 1A). Experimental groups received either a daily ip injection of CsA at a suboptimal dose of 10 mg/kg, or rD-mPGPtide (0.5 mg iv) on days 0, 3, and 6. The MST for mice treated with either rD-mPGPtide or CsA was 28 and 30 days, respectively. A combination of the two treatment regimens resulted in significant prolongation of survival (MST of 53 days) compared to the untreated group (*P* < 0.05). In addition, the survival of the combination treatment group was significantly prolonged in comparison to either treatment alone (*P* ≤ 0.04). Thus, rD-mPGPtide and CsA have an additive inhibitory effect on the development of GVHD across MHC barriers.

Combination of rD-mPGPtide and short-term CsA prophylaxis for GVHD. The inherent toxicity and persistent immunosuppression associated with CsA are major complications associated with long-term administration for the prevention of GVHD. In this regard, it was hypothesized that rD-mPGPtide could eliminate the need for long-term CsA administration. To test this notion in the same haploidentical BMT model as described above, GVHD was induced by the transplantation of 5 × 10^6 donor (B6 × DBA2)F$_1$ T cells, and mice were either left untreated, injected iv with rD-mPGPtide (0.5 mg, days 0, 3, 6), or administered CsA (20 mg/kg) ip daily for 8 weeks (long-term CsA; Fig. 1B). Two additional experimental groups received CsA either alone (20 mg/kg) ip daily for only 2 weeks (short-term CsA) or in combination with rD-mPGPtide (0.5 mg iv, days 0, 3, 6). The survival of the rD-mPGPtide-treated mice (MST of 18 days) was significantly enhanced (*P* ≤ 0.01) compared with that of the untreated group (MST of 8 days). Both CsA-treated groups exhibited enhanced survival compared with the untreated group as well (*P* ≤ 0.01). However, short-term CsA administration (MST of 43 days) was less effective than long-term CsA administration (MST of 61 days). The coadministration of rD-mPGPtide with short-term CsA therapy (MST of 59 days) significantly enhanced survival of these mice (*P* ≤ 0.04) compared with the short-term CsA alone. It should also be noted that the survival of the combined rD-mPGPtide and short-term CsA-treated mice was equivalent to that achieved with long-term CsA administration (*P* = 0.75).

DISCUSSION

It is apparent from our results that administration of rD-mPGPtide and CsA together can significantly inhibit T cell reactivity both *in vitro* and *in vivo*, compared with either agent alone. The additive effect these agents display would allow for the reduced utilization of the more toxic immunosuppressive compound (CsA) while still maintaining the high degree of recipient sur-
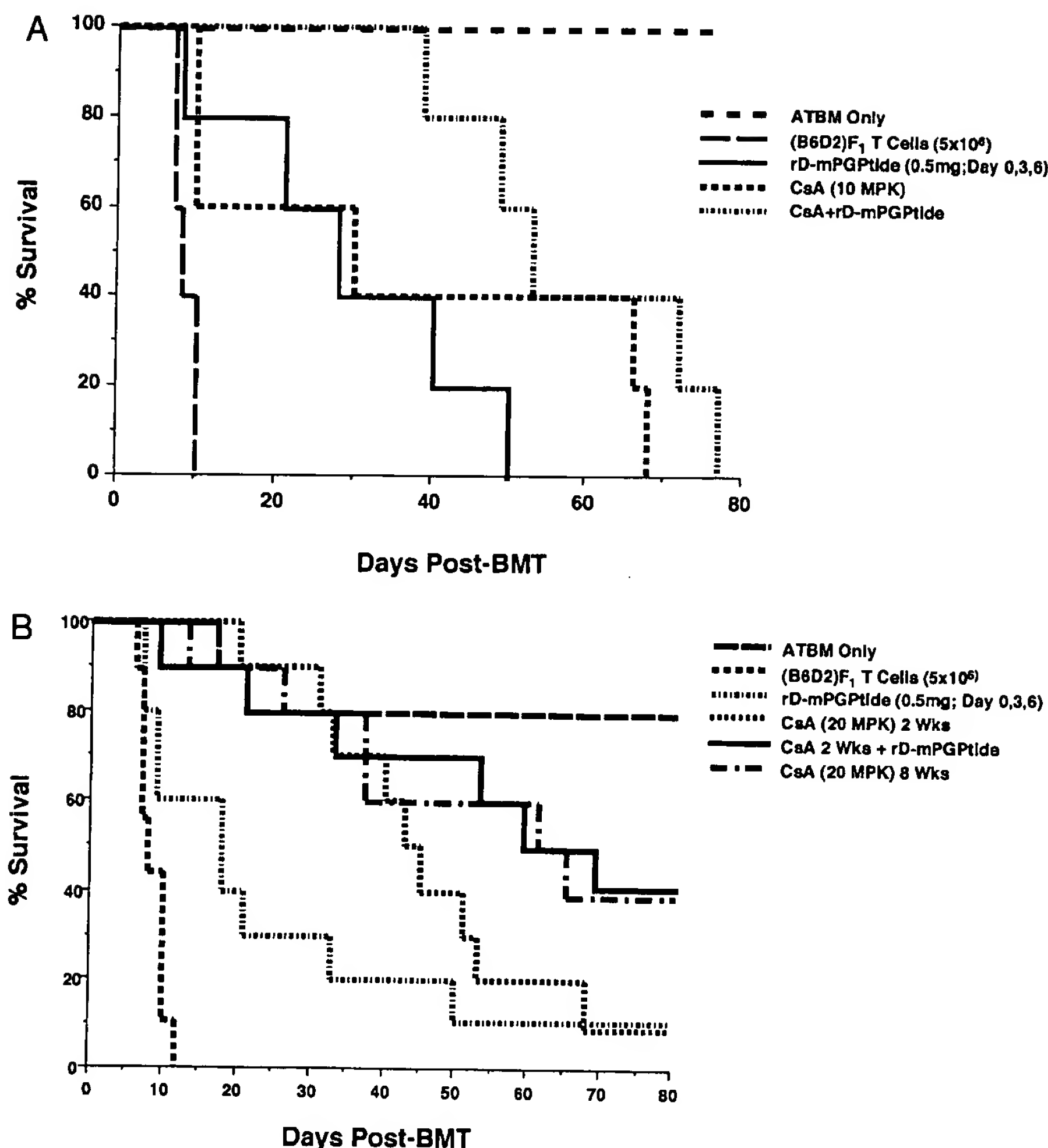
FIG. 1. Effect of treatment with rD-mPGPtide and CsA on survival of mice undergoing GVHD across a MHC-haploidentical barrier. (B6 × CBA)F₁ mice were lethally irradiated (950 cGy) and transplanted with allogeneic (B6 × DBA2)F₁ ATBM (2 × 10⁶) cells alone, or with unseparated donor T cells (5 × 10⁶) as indicated. (A) Recipients of T cells were either left untreated (positive GVHD control), injected iv with rD-mPGPtide (0.5 mg) every third day between days 0 and 6, injected ip with CsA (10 mg/Kg) daily, or given both regimens as indicated in the figure. The data are representative of three separate experiments and 5 mice were utilized per group. (B) Recipients of T cells were either left untreated (positive GVHD control), injected iv with rD-mPGPtide (0.5 mg) every third day between days 0 and 6, injected ip with CsA (10 mg/Kg) daily for 2 or 8 weeks, or given combinations of both regimens as indicated in the figure. The data are composites from two separate experiments, a total of 10 mice were utilized per group and significance was determined by non-parametric Wilcoxon signed rank analysis utilizing SYSTAT 5.2 software.

vival following BMT seen with continuous utilization of CsA. The probable differences in the mechanisms of action of these compounds allow for the speculation that these two agents could suppress T cells in a complimentary manner.

The mechanism by which CsA inhibits T cell responses via inhibition of IL-2 production is well known and can be partially blocked by the addition of exogenous IL-2. While the exact mechanism of rD-mPGPtide-mediated immunosuppression is still unclear, we previously demonstrated that the inhibition induced by rD-mPGPtide

is not overcome by the addition of exogenous IL-2 (8), thus making the combined immunosuppressive effect more resistant to circumvention by cytokine rescue. Furthermore, rD-mPGPtide is an analog of the murine CD4 molecule and has demonstrated little activity against CD8⁺ T cell responses. CsA is not specific to either T cell subset and thus will suppress CD8⁺ T cell responses. Thus, combining CsA with the peptide allows for inhibition of both T cell subsets which contribute to GVHD. We have previously postulated the rD-mPGPtide acts on T cells by disrupting the formation of the multimeric

CD4 complexes formed on the surface of the T cell upon TCR ligation thus preventing the proper cosignaling required for fulminant activation. Since it has been demonstrated that CsA inhibits the TCR signaling pathway by binding to calcineurin, the combination of the two agents would disrupt both the primary and secondary signaling mechanisms required for T cell activation. The result of inhibiting two of the major signal pathways simultaneously could result in the enhanced immunosuppressive activity seen.

The data thus far suggest only an additive effect of these two agents on T cell responses and more experimentation is required to demonstrate a synergistic effect of the compounds. In any case, the CD4-CDR3 peptide is indeed compatible with CsA treatment and in fact enhances its efficacy in preventing the development of GVHD following allogeneic BMT.

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Inhibitory Effect of a CD4-CDR3 Peptide Analog on Graft-Versus-Host Disease Across a Major Histocompatibility Complex-Haploidentical Barrier

By Robert M. Townsend, Constance Briggs, Joseph C. Marini, George F. Murphy, and Robert Korngold

A structure-based designed peptide has been engineered to exhibit the same molecular surface as a portion of the CDR3-like region in domain 1 of the murine CD4 molecule. Earlier in vitro experiments indicated that this analog, known as rD-mPGPtide, inhibited T-cell proliferation in mixed lymphocyte reactions and blocked activation of both normal CD4⁺ T cells and T-cell lines after T-cell receptor triggering. In addition, rD-mPGPtide proved to be a potent inhibitor in vivo of CD4⁺ T-cell–mediated experimental allergic encephalomyelitis disease in the SJL mouse model. In this current report, we have evaluated the potential of rD-mPGPtide for suppressing the development of graft-versus-host disease (GVHD) in an irradiated major histocompatibility complex (MHC)-haploidentical murine bone marrow transplantation (BMT) model [(B6 × DBA/2)F₁ → (B6 × CBA)F₁ (950 cGy)]. Our results indi-cated that early administration of rD-mPGPtide was effective in the inhibition of alloreactive responses of the donor T cells against the host and thus delayed or prevented the onset of GVHD. The median survival time of animals treated with rD-mPGPtide was enhanced as much as four-fold with as little as a single dose of peptide at the time of transplant. Decreased alloreactivity was indicated by phenotypic and functional analysis of positively selected thoracic duct lymphocytes 4 days after transplant and by histopathological examination of skin and gastrointestinal tissue samples 4 weeks later. Therefore, the administration of a CD4-CDR3 peptide is an efficacious approach against the development of GVHD during allogeneic BMT.

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$\mathbf{A}$LLOGENEIC BONE MARROW transplantation (BMT) is currently being used as a treatment for a number of disease states including several types of leukemia, aplastic anemia, and severe combined immunodeficiency, among others.[1] The major complications that impede the overall success of this treatment include the development of graft-versus-host disease (GVHD), marrow graft rejection, chronic immuno-incompetence, and leukemic relapse (in the case of BMT for the treatment of leukemias). Acute and chronic GVHD is caused by residual mature donor T cells in the bone marrow graft and leads to significant morbidity and mortality.[2] Removal of the mature T cells from the graft before engraftment reduces or prevents GVHD; however, this T-cell depletion also leads to reduced engraftment along with increased leukemia relapse rates.[3-5] These observations suggest the importance of a T-cell component in a successful BMT, although it is not clear whether GVHD reactive T cells can be separated completely from either the antileukemia effect or from enhanced hematopoietic engraftment. This question can only be approached by highly selective means of inhibiting those host-allospecific GVHD-reactive T cells, while allowing for the potential development of antileukemia-specific responses and for protection from opportunistic infections.

The CD4 molecule on the surface of helper T cells, in association with the T-cell receptor (TCR)-CD3 complex that recognizes specific antigen in the context of MHC class II, plays a critical role in the transmembrane and intracellular signaling pathways required for T-cell activation.[6-11] It has been well-established in murine models that CD4⁺ T cells are capable of mediating GVHD, primarily across MHC class II barriers,[12,13] but also in some cases with minor histocompatibility antigenic differences.[14,15] Inhibiting CD4⁺ T-cell responses by treatment of recipient mice with monoclonal antibodies (MoAb) directed against the CD4 molecule has effectively decreased the incidence of GVHD following BMT.[16-18] However, MoAb therapy has several limitations for potential clinical use, including but not limited to total subset depletion and immunogenicity of the MoAb itself.[19-21]

In previous reports, we have described the design and production of a peptide that specifically mimics the CDR3-like region in the D1 immunoglobulin domain of the murine CD4 molecule.[22,23] This peptide analog consists of thirteen amino acids (CELENRKEEPGPC) taken from the p86-94 sequence of the CD4 molecule with the addition of a proline-glycine-proline-cysteine sequence to the carboxyl terminus to allow cyclization and tertiary structural constraint. In order to make the peptide more resistant to protease degradation, it was synthesized with D-amino residues, necessitating the reversal of the amino acid order so that side chain presentation would be similar to the native molecule.[23] As a result of these adaptations, this CD4 peptide analog is referred to as reverse D amino acid mouse proline-glycine-proline peptide (rD-mPGPtide). The rD-mPGPtide is neither T-cell subset depletive nor immunogenic and thus has advantages over the use of anti-CD4 MoAb.

Our earlier reports documented that rD-mPGPtide is a potent inhibitor of certain types of CD4⁺ T-cell–mediated immune responses both in vitro and in vivo. In this report, we demonstrate the potential of rD-mPGPtide for inhibiting the in vivo alloreactive responses associated with the onset of GVHD in a major histocompatibility complex (MHC) haploidentical murine BMT model (B6 × DBA/2)F₁ → (B6 × CBA)F₁ (950 cGy). In this model, the donor and recipient mice possess both class I and class II differences. Injection

of irradiated (B6 × CBA)F₁ mice with a donor bone marrow inoculum supplemented with either 5 × 10⁶ unseparated or 1 × 10⁶ CD4⁺ enriched donor T cells induces an acute form of GVHD, which leads to fatality within 2 to 3 weeks. We show here that administration of rD-mPGPtide to recipient mice at varying times within the first week of transplantation significantly increased the median survival time of mice undergoing GVHD.

MATERIALS AND METHODS

Mice. Mice, (B6 × DBA/2)F₁, [(B6D2)F₁ (H2ᵇ/ᵈ)] and (B6 × CBA)F₁ [(B6CB)F₁ (H2ᵇ/ᵏ)], were purchased from the Jackson Laboratory (Bar Harbor, ME). Male mice were used as donors between the ages of 7 to 12 weeks and as recipients between the ages of 9 to 16 weeks. Mice were kept in a sterile environment in microisolators at all times and were provided with acidified water and autoclaved food.

Media. Buffered saline solution (BSS) supplemented with 0.1% bovine serum albumin (BSA) (Hyclone, Logan, UT) was used for all in vitro manipulations of the donor bone marrow and lymphocytes. For injection, cells were resuspended in BSS alone. RPMI 1640 (Mediatek, Herndon, VA) supplemented with 10% fetal calf serum (FCS) (Sigma, St Louis, MO) and 10 U/mL glutamine, 10 U/mL penicillin and streptomycin, and 0.05 mmol/L β-mercaptoethanol (Mediatech) was used for all in vitro mixed lymphocyte responses.

Peptides. The peptides were designed as previously described,[24] synthesized on an Applied Biosystems 430A peptide synthesizer (Foster City, CA) using standard Fmoc chemistry, refolded to enrich for intramolecular disulfide bonding, and purified by HPLC (Waters 600E system controller, Waters 490E programmable multi-wavelength detector; Millipore Corp, Bedford, MA) before use. The sequences of the synthesized peptides were as follows: rD-mPGPtide (CPGPEEKRNELEC, all D-amino acids) and scrambled rD-mPGPtide (Scr-PGPtide; CEPKNELPERGEC, all D-amino acids). For treatment of GVHD, peptides were reconstituted in PBS and injected at the appropriate dose and time into mice intravenously (IV) in a volume of 0.25 mL.

Irradiation. All recipient mice received a 950 cGy exposure from a Gammacell ¹³⁷Cs source (116 cGy/min).

MoAb. Ascites fluid for anti–Thy-1.2 (J1j, rat IgM)[25] and anti-CD8 (3.168, rat IgM)[26] MoAb were used for cell preparations. In addition, goat antimouse IgG (whole molecule) antibodies were purchased from Cappel-Organon Teknika (Westchester, PA). Guinea pig serum prepared in our laboratory was used as a source of C for all MoAb treatments. For phenotypic analysis of cells by flow cytometry, anti-murine CD4 (FITC-conjugated or biotinylated, as appropriate), CD25, CD71, CD95 (all biotinylated), and rat IgG (FITC- or PE-conjugated, as appropriate) standard control antibodies were purchased from Pharmingen (San Diego, CA).

Preparation of cells. Bone marrow cells were obtained from the femora and tibiae of donor mice by flushing with BSS with 0.1% BSA. To prepare anti–Thy-1–treated (T-cell–depleted) bone marrow (ATBM), cells were incubated with J1j MoAb (at 1:100 dilution) and C (1:25) for 45 minutes at 37°C and were washed four times. T-cell enriched donor cell populations were prepared by treating pooled spleen and lymph node (LN) cells with: Gey's balanced salt lysing solution containing 0.7% NH₄Cl for removal of RBC, and panning on a plastic petri dish pre-coated with a 5 μg/mL solution of goat antimouse IgG for 1 hour at 37°C to remove B cells. These treatments resulted in populations of 90% to 95% CD3⁺ cells, as quantitated by flow cytometric analysis. Further purification of T cells into CD4⁺ cells was performed as described previously.[14] These

procedures resulted in highly purified populations of CD4⁺ cells (>90%) with no detectable presence of the inappropriate subset.

Flow cytometric analysis. In a 96-well plate, 2 × 10⁵ cells/sample were incubated and washed with BSS containing 1% fetal bovine serum (FBS) and 0.05% NaN₃ (FACS buffer). Antibodies, conjugated to either FITC or biotin, were added to the appropriate wells in a volume of 25 μL for 30 minutes at 4°C then washed three times in FACS buffer and fixed overnight at 4°C in PBS containing 1% paraformaldehyde. In the case of the biotin conjugated antibodies, PE-streptavidin (Caltag, San Francisco, CA) was added (1:100 dilution) before fixation with paraformaldehyde and incubated for an additional 30 minutes at 4°C, then washed three times in FACS buffer and fixed with paraformaldehyde. Samples were analyzed on a Coulter Epics Profile II (Coulter Corp, Hialeah, FL).

In vitro mixed lymphocyte reaction (MLR). Single cell suspensions of responder cells for the murine MLR were obtained from either spleen and lymph nodes or TDL, as indicated. Stimulator cells were obtained from the spleens of indicated mice, irradiated with 15 Gy and washed three times with medium. In a 96-well plate 4 × 10⁵ responder cells were incubated with 8 × 10⁵ stimulator cells or medium alone for the indicated period of time at 37°C, 5% CO₂. Cultures were incubated with 1 μCi [³H]TdR/well for the final 24 hours, harvested, and counted. The percent response was calculated in the following manner: experimental CPM [³H]TdR minus medium alone CPM)/(anti-(B6CB)F₁ only CPM [³H]TdR minus medium alone CPM). Responses indices were calculated as a ratio of the experimental CPM [³H]TdR to the anti-(B6D2)F₁ only CPM [³H]-TdR. When indicated, culture supernatants were removed and tested for cytokine production by CTLL bioassay, as previously described.[27] Briefly, 1 × 10⁴ CTLL cells in 25 μL medium were added to 100 μL of culture supernatant in a 96-well plate. Anti-murine interleukin-2 (IL-2) MoAb was added at 2 μg/mL in 25 μL to appropriate wells. Cells were cultured for 24 hours and incubated with 1 μCi [³H]TdR/well for the final 6 hours, harvested, and counted. Experimental results were compared with a standard curve of mIL-2 and IL-4. Statistical comparisons between experimental groups for proliferation responses were performed by the Student's *t*-test analysis, using SYSTAT 5.2 software.

Collection of thoracic duct lymphocytes (TDL). Anesthetized mice were cannulated 4 days after injection of 10⁷ (B6D2)F₁ CD4⁺ T cells by insertion of an Intramedic PE 50 tubule into a fistula perforated in the cysterna chylae, as previously described.[28] The mice were then placed on an apparatus which allows the mice exercise and access to food while they are being infused IV with physiologic saline. The lymph was collected for 8 to 10 hours in 15-mL tubes containing 2 mL RPMI 1640 medium supplemented with 10% FBS, 1 U/mL heparin, and kept at 4°C until assay.

Mortality assay for GVHD. Recipient mice were irradiated with 950 cGy and approximately 6 hours later were injected IV (in a maximum volume of 0.5 mL of BSS) with either 2 × 10⁶ donor ATBM cells alone, as a negative control, or a mixture of ATBM plus donor T cells, as indicated. Mice were checked daily for morbidity and mortality until the experiments were terminated at day 60 posttransplantation. Median survival times (MST) were calculated as previously described.[14] Statistical comparisons between experimental groups for mortality curves were performed by the nonparametric Wilcoxon signed rank analysis, using SYSTAT 5.2 software.

Histopathological analysis. Two mice per experimental group were killed on day 27 post-BMT and organs were removed and fixed with 4% paraformaldehyde. Ear skin and gut were then processed for embedding in paraffin. Paraffin sections (6 μ) were cut and stained with hematoxylin and eosin (H&E). Sections were examined microscopically as indicated for the presence of inflammatory infiltrates and dyskeratotic or necrotic cells.
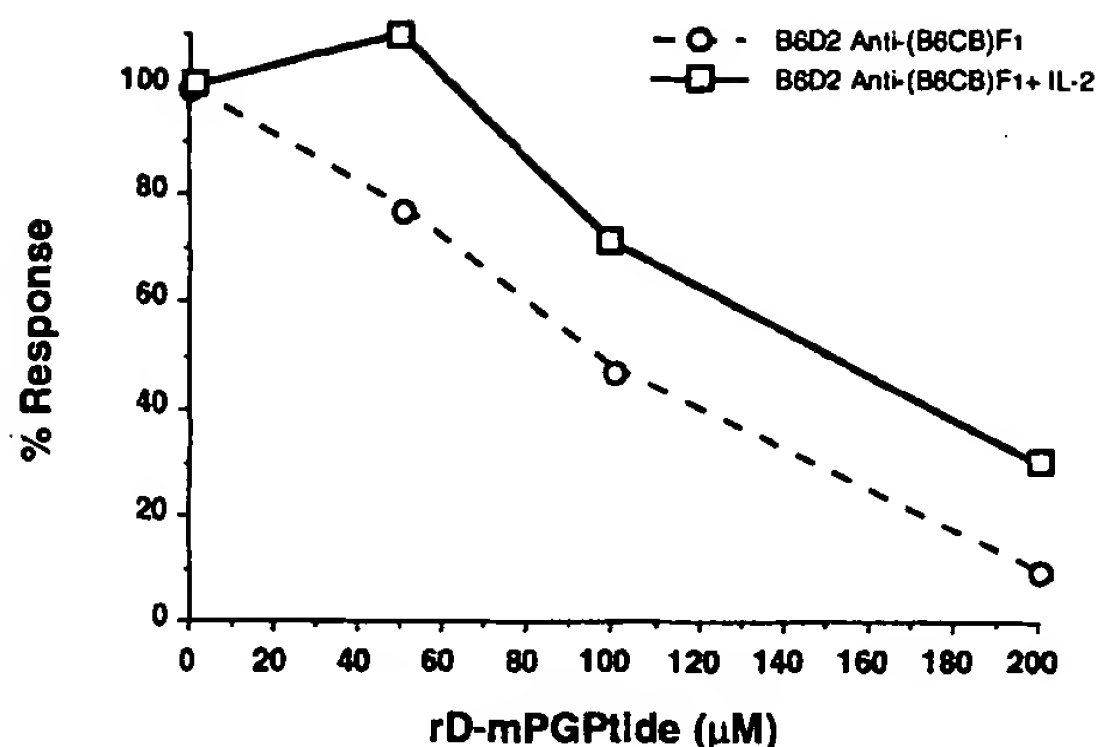
Fig 1. Inhibition of in vitro allogeneic MLR by rD-mPGPtide. The data shown are representative of three separate experiments and expressed as mean percent response [³H]TdR Incorporation of triplicate wells. The rD-mPGPtide was added at 200, 100, and 50 μmol/L to the appropriate wells and rhIL-2 was added at 100 U/mL to the appropriate wells.

RESULTS

rD-mPGPtide inhibits alloreactivity in vitro. Lymph node cells taken from (B6D2)F₁ mice were stimulated in vitro with irradiated (15 Gy) spleen cells from (B6CB)F₁ mice and the proliferative response was measured by [³H]-TdR incorporation on day 4. Titered concentrations of rD-mPGPtide (50 to 200 μmol/L) were added to appropriate wells in order to test the inhibitory properties of the peptide. As shown in Fig 1, the addition of rD-mPGPtide inhibited proliferation in a dose-dependent manner. Nearly 90% inhibition of the proliferative response was observed with the addition of 200 μmol/L of rD-mPGPtide and the 50% inhibitory concentration (IC₅₀) was 50 to 100 μmol/L. Furthermore, the presence of 100 U/mL of recombinant human IL-2 in the cultures failed to re-establish the proliferative response. It appeared that rD-mPGPtide could inhibit alloreactivity generated by this MHC-haploidentical strain combination in vitro, and thus had the potential to inhibit such immune responses in vivo, as well.

rD-mPGPtide inhibits acute GVHD directed across a MHC barrier. An acute form of GVHD was induced in lethally irradiated (950 cGy) (B6CB)F₁ mice by IV administration of 5 × 10⁶ (B6D2)F₁ T cells along with 2 × 10⁶ (B6D2)F₁ ATBM. In this GVHD model, the untreated mice exhibited 80% fatality by day 20 posttransplant with a MST of 13 days (Fig 2). Transplanted mice were also administered three different regimens of rD-mPGPtide treatment, including: (1) daily injections (0.5 mg/injection) from days 0 to 6 post-BMT; (2) alternate days during this same time period (days 0, 2, 4, 6); and (3) every third day (days 0, 3, 6). Treatment with these regimens of rD-mPGPtide increased the MST of these mice to 32, 30, and 28 days, respectively. The observed increases were statistically significant as compared with the untreated group (P < .03 for all of the rD-mPGPtide–treated groups), although there was little difference between the three different regimens among themselves (P > .05). With time, all of the peptide-treated mice that

received donor T cells eventually succumbed to GVHD. Both CD8⁺ and CD4⁺ T cells are likely to play a role in the development of GVHD in this strain combination due to the fact that the donor and recipient mice differ at both class I and class II MHC loci. Since the donor T cell subsets were unseparated, there was a potential development of a CD8⁺ T-cell–mediated GVHD, against which the rD-mPGPtide would be expected to have little effect.

rD-mPGPtide inhibits acute GVHD mediated by MHC allogeneic CD4⁺ T cells. To specifically evaluate the effect of rD-mPGPtide on CD4⁺ T cells during the GVHD response, an acute form of GVHD was again induced in irradiated (950 cGy) (B6CB)F₁ mice by administration of 1 × 10⁶ (B6D2)F₁ CD4⁺ T cells along with 2 × 10⁶ (B6D2)F₁ ATBM. As shown in Fig 3, the MST for those mice left untreated was 25 days. Mice treated with rD-mPGPtide on days 0, 3, and 6 exhibited a significant increase in the MST to >60 days post-BMT (P < .02). In this case, 82% of the mice treated with rD-mPGPtide survived for the duration of the experiment, as compared with 27% of the untreated GVHD mice and 91% of the control mice transplanted with only ATBM (P < .02). A single injection of rD-mPGPtide also resulted in a significant increase in the MST (P < .05) with 80% of the mice surviving past 60 days. A cyclized control scrambled peptide (Scr-PGPtide) was also tested (0.5 mg administered on days 0, 3, and 6) to ensure specificity of the rD-mPGPtide and did not significantly affect survival as compared with the untreated mice (P > .99), with only 33% surviving past 60 days (Fig 3). The body weights of surviving animals at the conclusion of the experiment (day 60) exhibited little differences between groups: 30.0 ± 1.0 g for the ATBM group, 27.9 ± 2.4 g for the GVHD group, 26.6 ± 5 g for the rD-mPGPtide day 0,3,6-treated group, 28.1 ± 1.3 g for the rD-mPGPtide day 0-treated group, and 26.6 ± 3.3 g for the Scr-PGPtide–treated group. These data suggested that a chronic form of GVHD was absent in these surviving mice. To examine the possibility that regulatory cells were being generated during peptide treatment, 1.25 × 10⁷ spleen and LN cells from rD-mPGPtide–treated (B6CB)F₁, mice more than 80 days posttransplantation of 10⁶ (B6D2)F₁ CD4⁺ T cells were transferred to de novo-irradiated (B6CB)F₁ mice undergoing GVHD by the same conditions. No enhancement of survival of the mice receiving adoptively transferred lymphoid cells (MST = 16 days; percent survival = 20%) was observed, as compared with the GVHD mice receiving just donor CD4⁺ T cells (MST = 13 days; percent survival = 0%).

Histopathological analysis. To evaluate the peptide's effect on the clinical manifestation of GVHD in the target tissues, histology sections were prepared 27 days post-BMT from rD-mPGPtide–treated or untreated mice undergoing GVHD. Tissue samples were taken from ear skin and the gastrointestinal tract and examined for morphological changes such as swelling, cellular damage, and the presence of inflammatory infiltrates. In the ear skin and small intestinal tissue of the untreated GVHD mice (Fig 4B and E) there was significant inflammatory infiltration in comparison with the ATBM controls (Fig 4A and D). Numerous dyskeratotic and necrotic cells were also observed in the epidermal layers
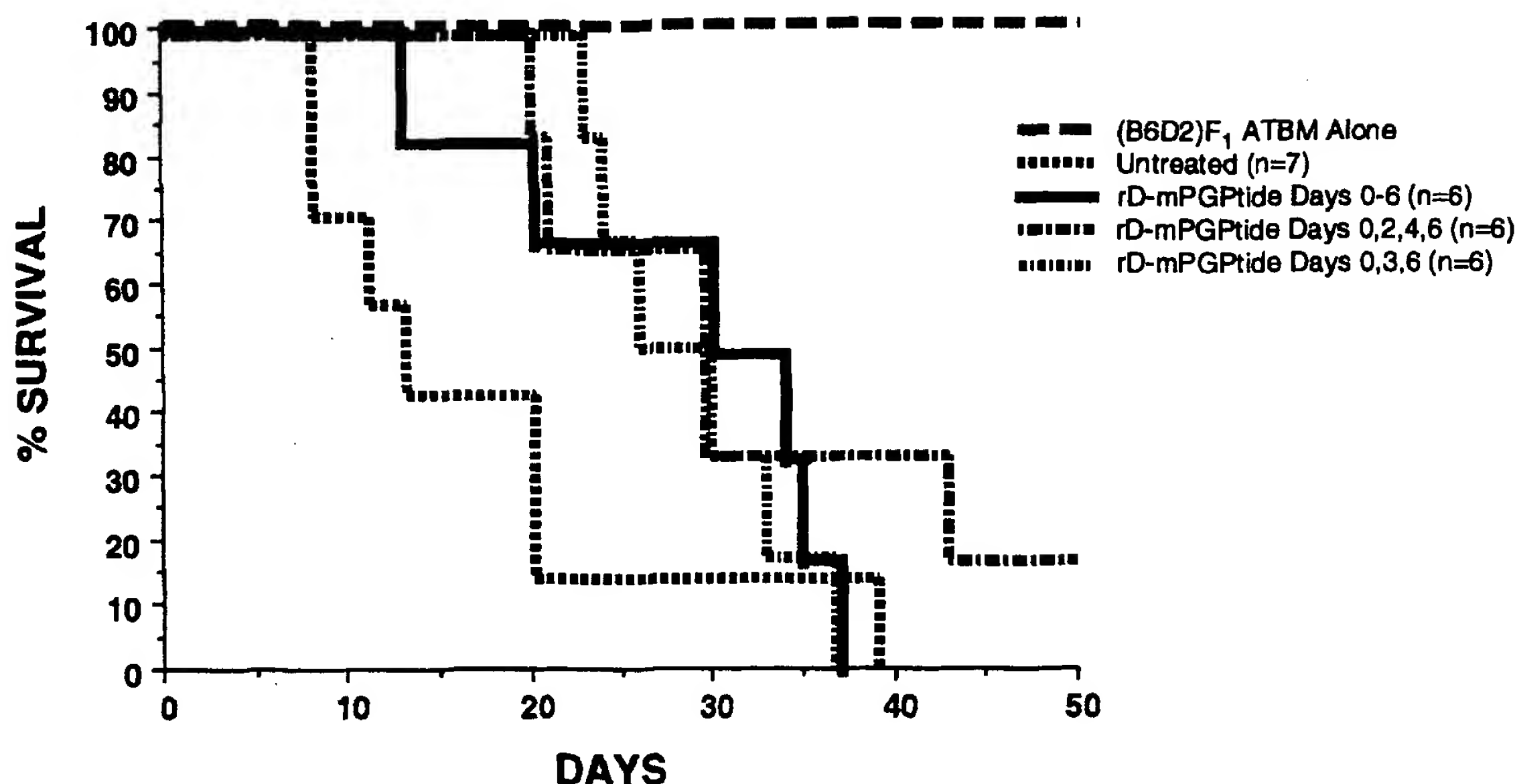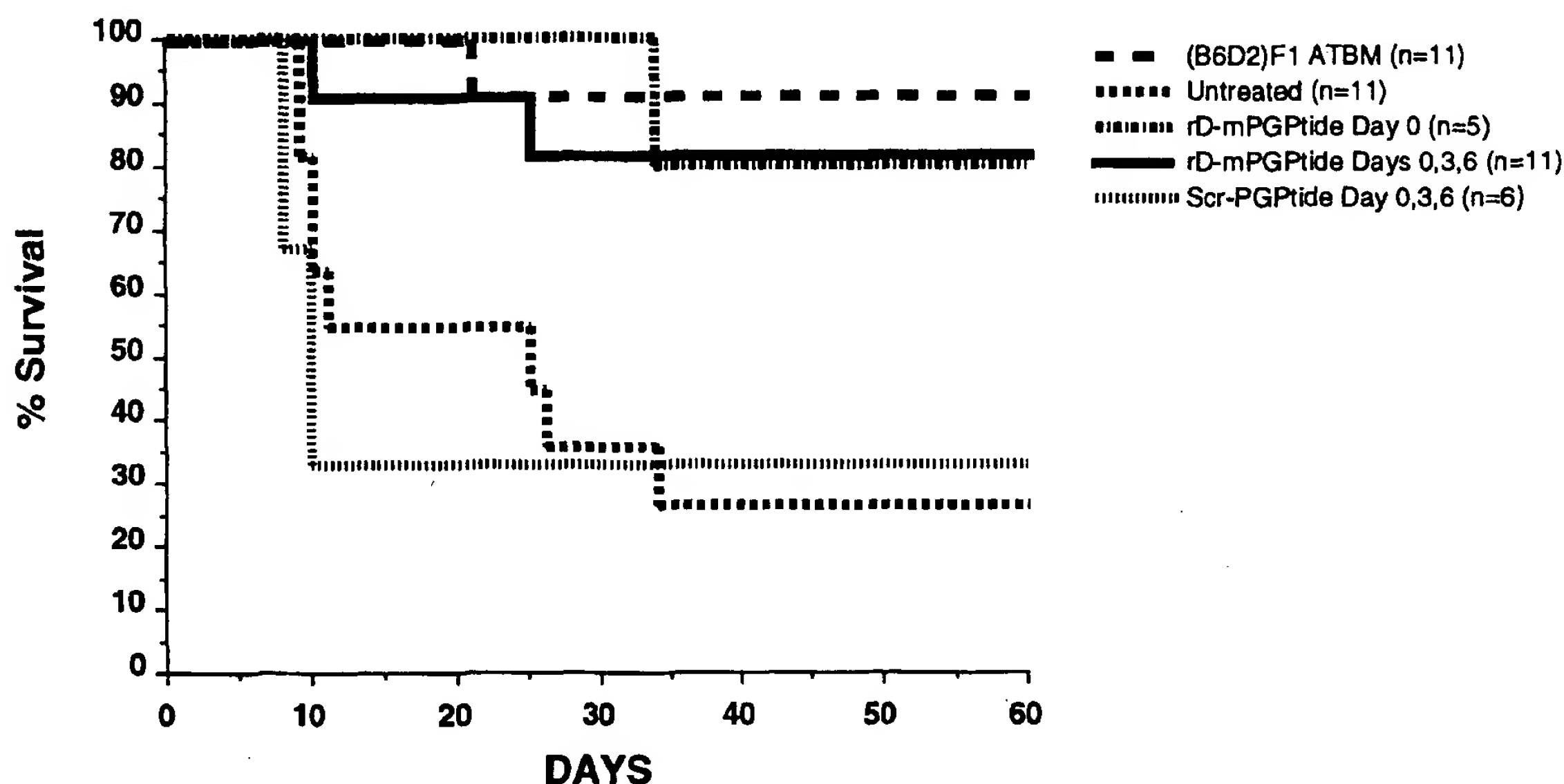
Fig 2. Survival of mice undergoing GVHD across a MHC-haploidentical barrier is enhanced by varying treatments with rD-mPGPtide. (B6CB)F₁ mice were lethally irradiated (950 cGy) and transplanted with allogeneic (B6D2)F₁ ATBM (2 × 10⁶) cells alone, or with unseparated donor T cells (5 × 10⁶). Recipients were either left untreated (positive GVHD control), injected IV with rD-mPGPtide (0.5 mg) daily, every other day, or every third day between days 0 and 6. The data are representative of three separate experiments and the numbers of mice used per group are indicated in the figure.

Fig 3. Survival of mice undergoing CD4⁺ T-cell-mediated GVHD across a MHC-haploidentical barrier is enhanced by treatments with rD-mPGPtide. (B6CB)F₁ mice were lethally irradiated (950 cGy) and transplanted with allogeneic (B6D2)F₁ ATBM (2 × 10⁶) cells alone, or with CD4⁺ T cells (1 × 10⁶). The rD-mPGPtide (0.5 mg) was administered IV on either day 0 alone or on days 0, 3, and 6. Scr-PGPtide (0.5 mg) was injected on days 0, 3, and 6 and had no significant effect on survival. Data are pooled from two separate experiments and the total numbers of mice used per group are indicated in the figure.
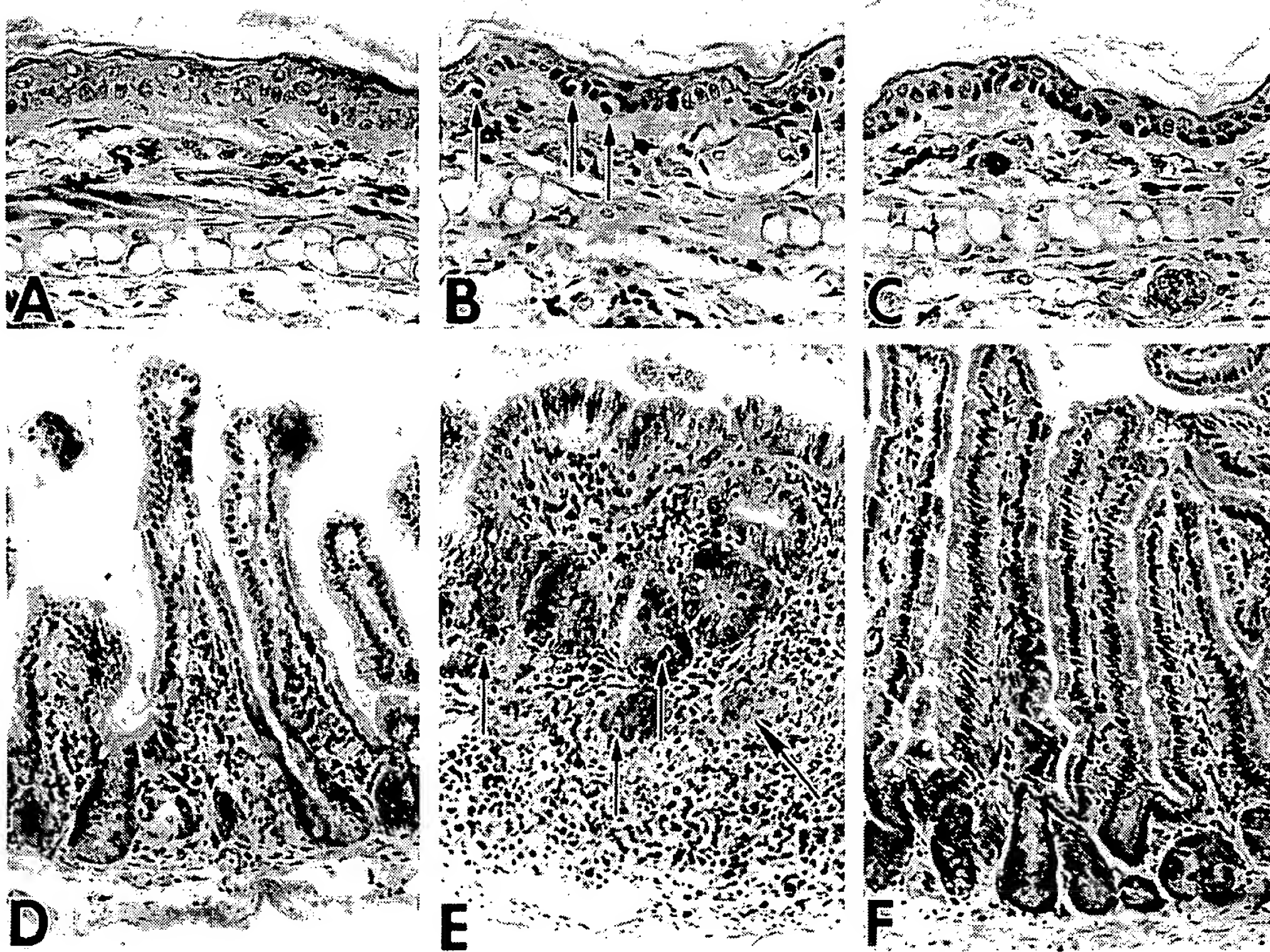
Fig 4. Ear skin (A through C) and small intestine (D and E) of irradiated (950 cGy) (B6CB)F₁ mice 27 days after the transplantation of either (B6D2)F₁ ATBM (2 × 10⁶) alone (A and D) or with 1 × 10⁶ (B6D2)F₁ CD4⁺ T cells, and left untreated (B and E) or rD-mPGPtide–treated (0.5 mg) on days 0, 3, and 6 (C and F). Two mice per group were examined. The epidermal layer and intestinal epithelium of mice receiving only ATBM (A and D) were devoid of cellular injury, whereas numerous dyskeratotic and necrotic cells (arrows) were observed in the untreated positive GVHD controls (B and E). Also note the cellular infiltrate in lamina propria of positive control intestine (E). Peptide-treated animals failed to exhibit significant skin (C) and gut (F) pathology, and resembled ATBM controls. (Final magnification: A-C, ×800; D-F, ×500).

of both the skin and gut, suggesting GVHD-related cell death. In contrast, the tissue samples from the rD-mPGPtide–treated mice (Fig 4C and F) exhibited limited inflammatory infiltrates and significantly fewer dyskeratotic cells. The differences between the groups were quantitated by counting the number of dyskeratotic cells per linear millimeter of epidermis (Fig 5). The samples from peptide-treated mice displayed a two- to three-fold decrease in the frequency of dyskeratotic cells as compared with those from the GVHD control mice. These combined data suggested that prophylactic treatment of transplanted mice with rD-mPGPtide significantly reduced the clinical manifestations of GVHD, as evidenced by histological sampling.

Effect of rD-mPGPtide on activation antigen expression in vivo—day 4 TDL. To begin investigating the mechanism by which rD-mPGPtide prevents the onset of GVHD, we examined the cell surface of CD4⁺ T cells from transplanted mice for the expression of activation antigens including IL-2-receptor (IL-2R; CD25), Fas (CD95), and Transferrin-receptor (Tr-R; CD71). Irradiated (950 cGy) (B6CB)F₁ mice were transplanted with 10⁷ (B6D2)F₁ CD4⁺ T cells, and on day 4 posttransplant their thoracic ducts were cannulated,
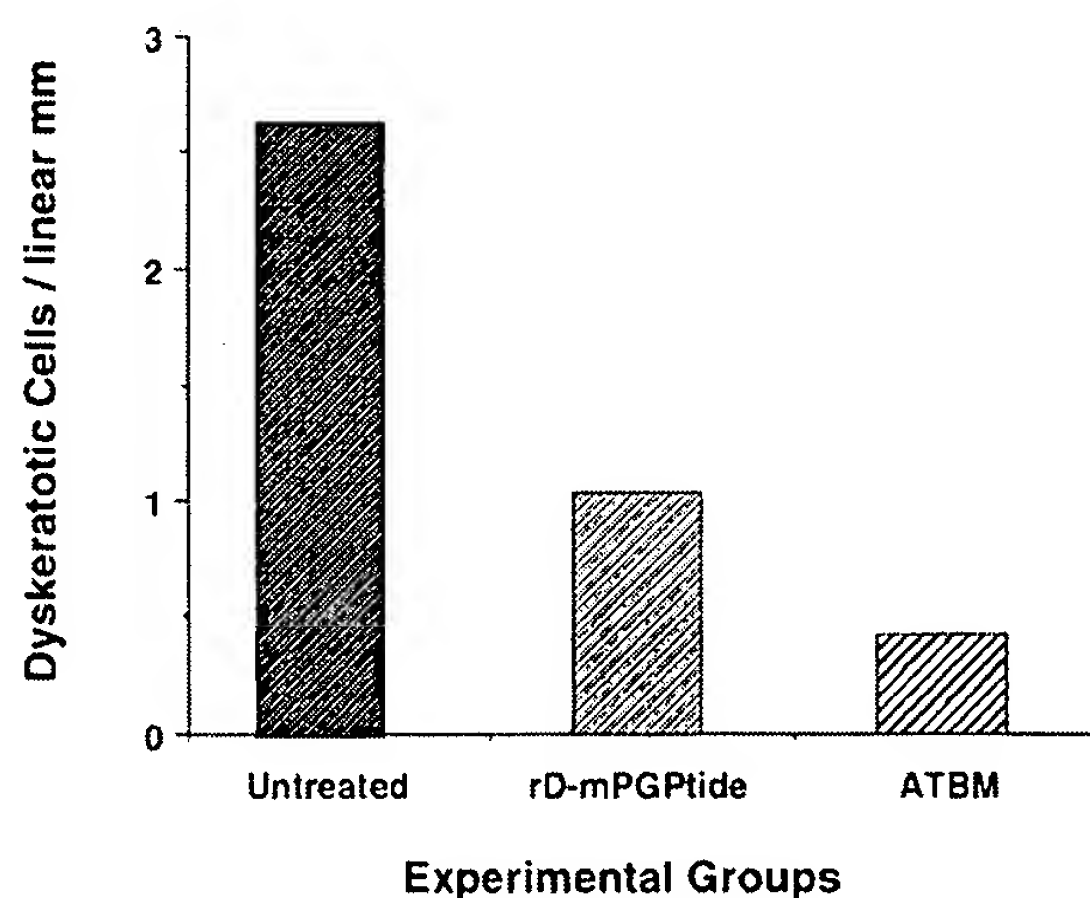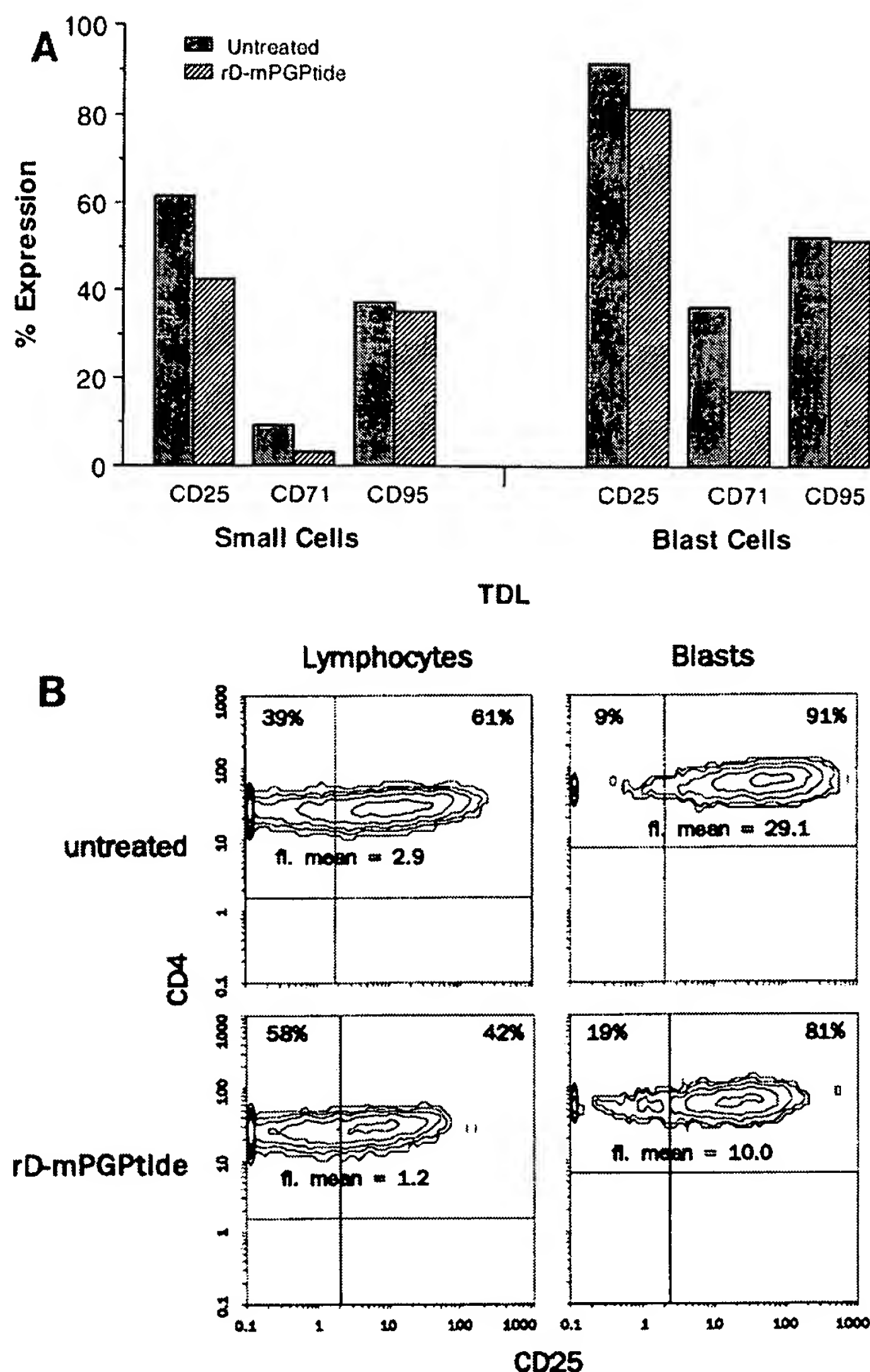

Fig 5. Dyskeratotic cells in the epidermal layer of ear skin were quantitated (#/linear mmol/L) for the same tissue samples as described in Fig 4, A-C. Approximately 10 linear mmol/L were counted in each ear specimen (two mice per group).

Fig 6. Activation phenotype of TDL cells collected from mice undergoing GVHD is altered by treatment with rD-mPGPtide. (B6CB)F₁ mice (five mice per group) were lethally irradiated (950 cGy) and transplanted with allogeneic (B6D2)F₁ CD4⁺ T cells (1 × 10⁷). Recipients were either left untreated or rD-mPGPtide (0.5 mg) was administered IV on days 0 and 3. Mice were cannulated on day 4 and TDL cells collected over an 8- to 10-hour period. (A) Expression of CD25, CD71, and CD95 as a percentage of the small lymphocytes and the blasting lymphocytes. (B) Flow cytometric profile of the CD4⁺ TDL cells for expression of IL-2 receptor (CD25). The negative control samples for the control and peptide-treated lymphocytes were 0% positive for CD25 with a mean fluorescence of 0.153 and 0.158, respectively. The negative control samples for the control and peptide-treated blast cells were 9% and 7% positive for CD25, respectively, with a mean fluorescence of 0.226 and 0.232, respectively.

TDL were collected over an 8 to 10 hour period, and flow cytometric analysis was performed on the retrieved cells. Transplanted mice (3 per group) were either left untreated or were injected IV with 0.5 mg rD-mPGPtide on days 0 and 3. The TDL collected from each group were exclusively donor-type CD4⁺ T cells; however, the flow yield of TDL collected from peptide-treated mice was significantly less than those of the untreated mice, 4 × 10⁵ cells/mL versus 7 × 10⁵ cells/mL, respectively. Both experimental groups of TDL contained a high percentage of blast-like cells (26% to 28%), and were analyzed as a separate population from the remaining small lymphocytes. As shown in Fig 6A, the percentage of TDL cells expressing the activation antigen CD25 was very high (90% for blast cells and 60% for non-blast cells) in the mice undergoing GVHD, suggesting that the

allogeneic donor CD4⁺ T cells were reacting to the host alloantigens. Treatment of the mice with rD-mPGPtide resulted in a 30% reduction in the percentage of non-blast cells expressing IL-2R and a 10% reduction in the percentage of blast cells expressing IL-2R. More notable, were the changes in the mean antigen expression of IL-2R on both the blast cells and the non-blast cells. Nearly a three-fold reduction was seen for both populations (Fig 6B). Tr-R expression was also affected in a similar manner; however, Fas appeared to be expressed at a level of 50% in both cell populations and remains unchanged in the peptide-treated group (Fig 6A).

Alloreactivity of GVHD TDL cells. As a measure of alloreactivity, MLR cultures were established using the TDL collected above as responder cells. These cultures were stim-
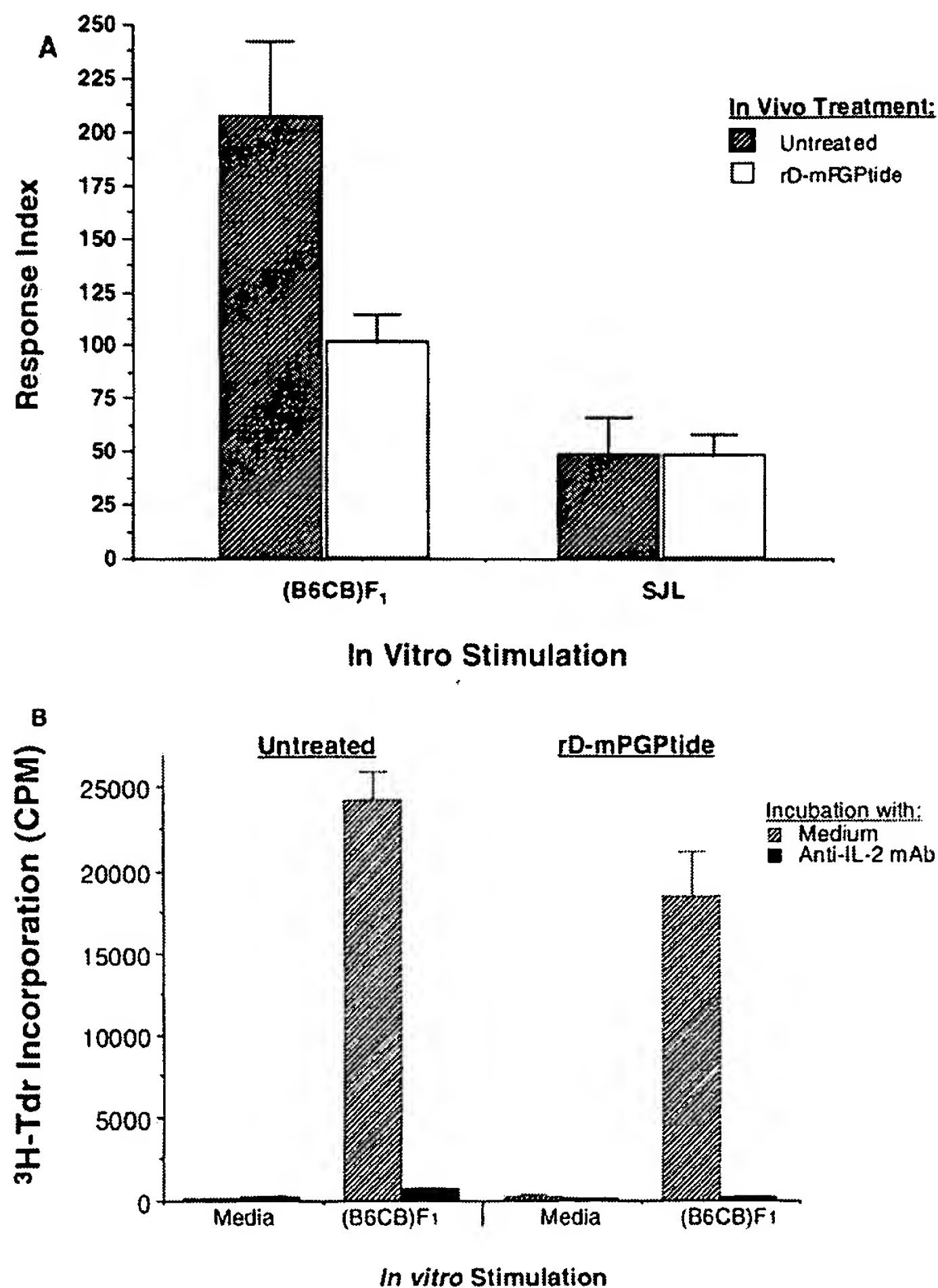
Fig 7. Alloreactivity of TDL cells collected from mice undergoing GVHD is reduced by treatment with rD-mPGPtide. The TDL cells were collected in the same manner as described in Fig 6 and the data are representative of three similar experiments. (A) MLR proliferation responses to irradiated host (B6CB)F₁ splenocytes were performed as described in the Materials and Methods section. Data is expressed as the response index of proliferation ± SEM. (B) CTLL proliferation supported by culture supernatants from TDL alloresponses. Data is expressed as CPM [³H]TdR incorporation ± SEM.

ulated with either irradiated (15 Gy) (B6CB)F₁ splenocytes or media alone for 48 hours and pulsed with [³H]TdR as a measure of proliferation for the final 24 hours. As shown in Fig 7A, [³H]TdR incorporation by TDL from untreated control mice was enhanced in response to (B6CB)F₁ stimulator cells, as compared with the syngeneic (B6D2)F₁ stimulator cells. TDL collected from the rD-mPGPtide–treated mice exhibited a 50% reduction in proliferation when incubated with the allostimulator cells as compared with the untreated TDL proliferation (P < .01). Furthermore, proliferation responses to third party alloantigens (SJL spleen cells) remains intact following peptide treatment (P > .05). These results suggested that proliferative responses to alloantigen are impaired in the peptide-treated mice. To further analyze this proliferative response, culture supernatants from another representative experiment were removed after 24 hours and analyzed for cytokine content by a CTLL bioassay. As shown in Fig 7B, supernatants from the untreated control TDL cultured with media alone were not capable of supporting CTLL proliferation. Incubation of TDL with (B6CB)F₁ stimulator cells significantly enhanced the CTLL prolifera-

tion of the culture supernatants. This proliferation was neutralized by anti–IL-2 MoAb suggesting little or no IL-4 production by the TDL in response to the alloantigen stimulation. The TDL from the rD-mPGPtide–treated mice demonstrated slightly reduced cytokine production (P > .01), which was neutralized by anti–IL-2 MoAb as well, as compared with the untreated TDL. These results suggested that the proliferation defect of the TDL from peptide-treated mice may not be entirely accounted for by a reduced capacity to produce IL-2.

DISCUSSION

In this report, we have described a novel therapeutic approach for the treatment of murine GVHD across a MHC barrier using a peptide analog of the CDR3 region of the murine CD4 molecule, rD-mPGPtide. Experiments performed both in vitro and in vivo have demonstrated a potential for rD-mPGPtide to affect immune responses to alloantigens. The inhibition of the alloreactive immune response was indicated in vivo primarily by the prolongation of survival of mice undergoing GVHD and reduced tissue destruction in

these mice. This enhanced survival was evident for GVHD induced by both unseparated T cells and purified CD4$^+$ T cells (Figs 2 and 3). Even a single injection of rD-mPGPtide at the time of transplant was effective in delaying the onset of disease mediated by CD4$^+$ T cells. The protective response of the rD-mPGPtide appears to be specific as the control scrambled peptide failed to exhibit significant enhancement of survival as compared with untreated mice in this model (Fig 3).

Upon comparing the two experimental conditions tested, unseparated T cells and purified CD4$^+$ T cells, it is apparent that the rD-mPGPtide was more effective in preventing the GVHD induced by the purified CD4$^+$ T cells. We hypothesize that in the case of the unseparated T cells, the rD-mPGPtide has limited effect on the CD8$^+$ T-cell-mediated component of GVHD directed to MHC class I antigens, a large portion of which may actually be CD4-independent.[13] Yet, even under these arduous conditions, the CD4-CDR3 peptide could prolong the survival time of recipient mice by at least 2 weeks.

Based on the animal survival data, it is apparent that the alloreactive (B6D2)F$_1$ CD4$^+$ T cells from the donor are less potent at mediating GVHD in the rD-mPGPtide-treated recipient. The anti-host specific donor T cells, themselves, are most likely rendered dysfunctional in their ability to respond to alloantigen. This is supported by several observations, including the reduction of in vitro alloreactivity and activation antigen expression by the positively selected TDL collected from mice treated with rD-mPGPtide. In vitro alloresponses and cytokine production were evident in the TDL of the mice undergoing GVHD (Fig 7, A and B); however, the TDL from the peptide-treated mice exhibited a 50% reduction in the proliferative response to alloantigen, but only a 15% reduction in IL-2 production. This reduced proliferative capacity could be due to either an inability of the T cells to recognize alloantigen or the presence of fewer alloreactive T cells due to deletion and/or inhibition of expansion. However, these results suggest that the observed inhibition of proliferation is relatively independent of IL-2 production. Furthermore, expression of all three activation antigens tested (IL-2R, Tr-R, and Fas) were increased on the TDL of GVHD mice (Fig 6A), whereas the TDL from the rD-mPGPtide-treated mice exhibited a marked reduction in the expression of IL-2R and Tr-R, yet no reduction in the expression of Fas. These findings may hold a clue to the mechanism by which this CD4-CDR3 peptide analog mediates inhibition of GVHD. It has been shown previously that the expression of Fas is required for the induction of T-cell apoptosis on incomplete activation, while the lack of Fas expression leads to T-cell anergy.[29] The continued high expression of Fas on the TDL from the peptide-treated mice may allow for the induction of apoptosis of the allo-specific T cells on incomplete activation in the presence of peptide. Further investigation into the peptide mechanism is necessary to clarify this issue.

Several recent reports propose that alterations in the immune response can be induced which generate a protective effect against various immunologically based disorders. These approaches include the polarization in cytokine pro-

duction from Th1-like to Th2-like phenotypes,[30,31] potentiation of other specific cytokines (eg, TGFβ[32,33]), inhibition of T-cell trafficking to target organs,[34-38] or the generation of non-lethal regulatory cells in vivo.[39,40] The switching of cytokine phenotype from Th1 to Th2 has been reported to be induced by several agents including cytokines[41] and anti-CD4 MoAb.[42,43] The induction of a Th1 immune response in GVHD has been correlated with lethality and a switch in the response to a Th2 phenotype has been shown to significantly prolong survival.[30,31] In this regard, we investigated whether or not rD-mPGPtide was inducing such a cytokine switch leading to protection of the transplanted mice. TDL taken from these peptide-treated mice demonstrated no appreciable difference in the IL-2 and IL-4 production profiles when stimulated ex vivo by alloantigen (Fig 7B). Therefore, our data do not support a rD-mPGPtide-induced cytokine switch phenomenon in alloreactive T cells during the development of GVHD to account for the inhibitory effects of the peptide.

In order for the T cells to cause GVHD, they must be able to traffic to the appropriate target organs including the skin, gut, and liver. Inhibiting the trafficking of these alloreactive T cells could also lead to a reduction in the symptoms of GVHD. The expression of adhesion molecules such as CD44, ICAM-1, VCAM-1, and LFA-1 on the surface of T cells play an important role in controlling the trafficking of these cells to their target tissues.[35,44,45] The histopathological analysis of mice undergoing GVHD suggested that rD-mPGPtide was inhibiting the infiltration and subsequent destruction of epithelial tissue in the skin and gastrointestinal tract (Fig 4). In addition to the possible inhibition of proliferation and expansion of alloreactive T cells, this apparent inhibition of trafficking could also be a result of decreased expression of adhesion molecules on the surface of the activated T cells or insufficient upregulation of the ligand molecules on vascular tissues. These possibilities are currently under investigation.

The generation of regulatory cells controlling immune responses in various mouse models of autoimmunity has been previously reported.[39,40,46] Adoptive transfer of these cells from protected animals to unprotected autoimmune-prone animals can lead to protection from disease and is one way of demonstrating the existence of these regulatory populations of cells. We examined this possibility by transferring spleen and lymph node cells from rD-mPGPtide-treated (B6CB)F$_1$ mice more than 80 days posttransplantation of 10^6 (B6D2)F$_1$ CD4$^+$ T cells to de novo-irradiated (B6CB)F$_1$ mice undergoing GVHD by the same conditions. We observed no enhancement of survival of the mice receiving adoptively transferred lymphoid cells as compared with the GVHD mice receiving just donor CD4$^+$ T cells. Thus, it appears that the generation of a regulatory population of cells is not responsible for the observed protective effect of rD-mPGPtide in these mice undergoing GVHD.

As a therapeutic modality, the rD-mPGPtide peptide mimic of the CD4-CDR3 molecular site appears to be an effective agent for the prevention of GVHD. We believe that the peptide primarily affects the alloreactive T cells that are being activated early after transplantation, rendering them

incapable of inducing GVHD. Results from recent EAE studies[47] with the peptide provide strong evidence that inhibition is highly specific for CD4+ T-cell responses to antigens present at the time of exposure to the rD-mPGPtide. One, 2, or 14 days after treatment, there is no diminution in any of the lymphoid cellular compartments, including the CD4+ T-cell subset. In addition, lymph node T cells are fully functional in their capacity to respond to both recall antigens and to third-party alloantigens. The half-life retention of peptide in serum in mice is approximately 25 minutes and responsiveness to any type of antigen stimulation is significantly inhibited for up to 6 hours after administration, but has virtually no effect by 12 hours. Noting this short window of effect of the CD4-CDR3 peptide, it is a most intriguing possibility that if administered only within the first week of transplant, the peptide could leave the remaining non-alloreactive CD4+ T-cell population intact for subsequent development of responses to opportunistic infections or potential leukemic relapse. Further studies are planned to clarify these issues.

ACKNOWLEDGMENT

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